

**The Effect of Subinhibitory Concentrations
of Antibiotics on Virulence Factors of
Staphylococcus aureus Biofilms**

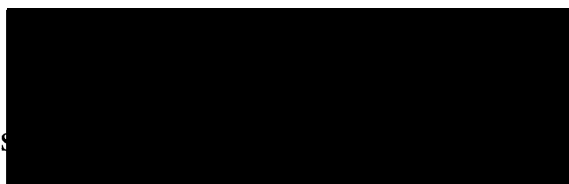
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I certify that this is a true and accurate version of the thesis approved by the
examiners

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Abstract

Staphylococci are normal colonizers of human and animal skin and mucous membranes. However, *S. aureus* is one of the most pathogenic bacteria isolated from humans. *S. aureus* produces numerous virulence factors that are responsible for the pathogenicity of this microorganism. Exposure of bacteria to sub-MICs of antibiotics is often encountered clinically, therefore, the effect of sub-MICs on staphylococcal virulence has been well studied on planktonic forms. Nevertheless, few studies have investigated the alteration of biofilm virulence under sub-MIC conditions.

In this study, after several attempts to support the growth of *Staphylococcus aureus* in chemically defined media containing the basic nutrient requirements, addition of seven amino acids improved the growth yield. However, staphylococcal growth in biofilm required a complex medium, such as brain heart infusion, which was used in the study. The effect of 1/16 MIC of selected antibiotics representing three different groups (cefalexin, a cell wall inhibitor; ciprofloxacin, a DNA replication inhibitor and roxithromycin, a protein synthesis inhibitor), was investigated on the virulence of *S. aureus* NCTC 11962 biofilms grown in Sorbarod filters. Protease secretion, coagulase production, ability to form biofilm and the superantigen (toxic shock syndrome toxin-1 (TSST-1)) production were evaluated in challenged and control biofilms, eluates and supernatants. Moreover, since sub-MICs of antibiotics have the potential to alter virulence and interfere with metabolic processes of the bacteria, the proliferative effect of TSST-1 was evaluated to determine any modulation in its activity.

SDS-PAGE and 2D-PAGE of biofilm and eluate cells in addition to eluate supernatants exhibited remarkable differences in protein profiles among challenged and control biofilms. Such protein shifts would imply in part, alterations in biofilm virulence.

Proteases were not detected in the eluates of the treated or control biofilms. Coagulase production was reduced in eluates exposed to roxithromycin when compared to control, while TSST-1 production was reduced in biofilms exposed to cefalexin and to a lesser extent to ciprofloxacin. On the other hand, cefalexin has been shown to induce biofilm formation at a wide range of sub-MICs.

TSST-1 was purified from eluate supernatants of treated biofilms and control and the proliferative activity of 10-fold serial dilutions of the toxins on mouse splenocytes were tested using MTS/PMS assay. No significant difference in activity between the treated toxin and the control has been observed. However, the activity of the crude eluate supernatants has been found to be more active when compared to equivalent concentrations of purified TSST-1, an effect ascribed to the presence of enterotoxin A, another superantigen produced by the bacteria. An hypothesis to explain the inhibitory effect of cefalexin on TSST-1 production was also discussed.

The results obtained in this thesis, indicate that antibiotics could potentially interfere with the virulence of *S. aureus* biofilms, if concentrations of these agents fall below MIC of the infecting strain.

Declaration

I declare that this thesis has not been submitted for any academic award at this or any other university.

Signed



Date 17/04/2008

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(PBPs) which catalyze transpeptidation, carboxypeptidation and other reactions (Kozarich and Strominger, 1978). Four PBPs have been identified in *S. aureus*. Selective inactivation of each Penicillin Binding Protein will result in different effects on bacterial cells. For example, spheroplast formation or change in the bacterial shape (elongation, filamentation or the formation of spheroid cells) (Petri, 2001). In addition, it is postulated that there is a balance system between cell wall synthesis mediated by PBPs and wall hydrolysis, which is essential for cell wall growth. By binding to PBPs, β -lactams disrupt this balance causing cell autolysis (Petri, 2001). Cell autolysis may occur due to β -lactams triggering the action of cell wall autolytic enzymes (Blumberg and Strominger, 1974; Spratt, 1983).

Staphylococci confer resistance to β -lactam antibiotics by producing β -lactamase that hydrolyses the β -lactam ring, rendering the antibiotic inactive. The gene for β -lactamase is located on a transposable element on a large plasmid. The synthesis of this enzyme is upregulated when the bacteria are exposed to β -lactam antibiotics (Lowy, 2003). On the other hand, *S. aureus* strains, which are resistant to methicillin (a semisynthetic penicillin-related antibiotic, which is resistant to β -lactamase), were found to increase the production of β -lactamase or to reduce the affinity of β -lactam compound's binding to Penicillin Binding Protein (PBP) present in the cell wall. The reduced affinity could be a result of modifying the native PBP and or producing PBP 2a, which is encoded by chromosomal gene (Utsui and Yokota, 1985; Murakami *et al.*, 1987; Chambers, 1997). PBP 2a has 100 to 1000 times less affinity to β -lactams than normal PBPs (Chambers, 2006).

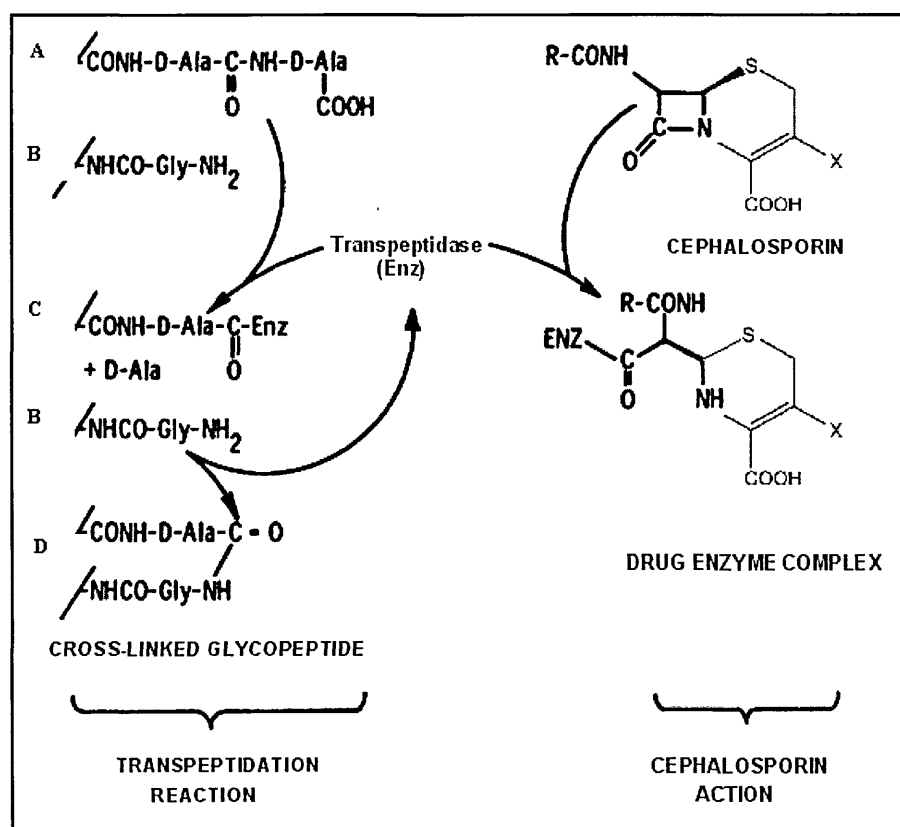


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1.2.2 Ciprofloxacin

Ciprofloxacin (Figure 1.2B) is a synthetic antibiotic belonging to the fluoroquinolone family. It is bactericidal and has broad spectrum activity against a wide range of microorganisms. Some reports have shown that the activity of ciprofloxacin is enhanced when used with other antimicrobials, such as aminoglycosides against *S. aureus* and *Pseudomonas* spp and cefotaxime or clindamycin against anaerobic bacteria. Ciprofloxacin can be administered orally, parenterally and topically (Sweetman, 2007).

Fluoroquinolones act by inhibiting bacterial DNA replication. To do this, they have two targets; DNA gyrase (which changes DNA supercoiling during replication) and topoisomerase IV (which decatenates or separates interlocked DNA strands to allow segregation of daughter chromosomes into daughter cells) (Drlica and Zhao, 1997). Fluoroquinolones form complexes with these enzymes on DNA (Figure 1.4), which block DNA replication (Hooper, 2002). In Gram-positive bacteria, resistance develops to these antibiotics via spontaneous chromosomal alterations. These mutations lead to changes in amino-acids in certain regions of the enzymes' subunits known as Quinolone-resistance-determining-region (QRDR), which will reduce the affinity of the enzyme to the drug (Hooper, 2002; Lowy, 2003). Since fluoroquinolones act on the two enzymes, single mutations on either one of them could result in reduced susceptibility to the drug, but not complete resistance. Nevertheless, fully resistant isolates of *S. aureus* are common where more than one mutation in each enzyme was found. In fact, single changes in Par C (a type of topoisomerase IV subunit) were found to be sufficient to produce resistance to Ciprofloxacin (Hooper, 2002). Another mechanism of resistance in *S. aureus* is by increasing the expression of multidrug resistance (MDR) pumps called NorA. These pumps reduce the concentration of antibiotics inside the cell (Piddock, 1999; Hooper, 2002; Lowy, 2003).

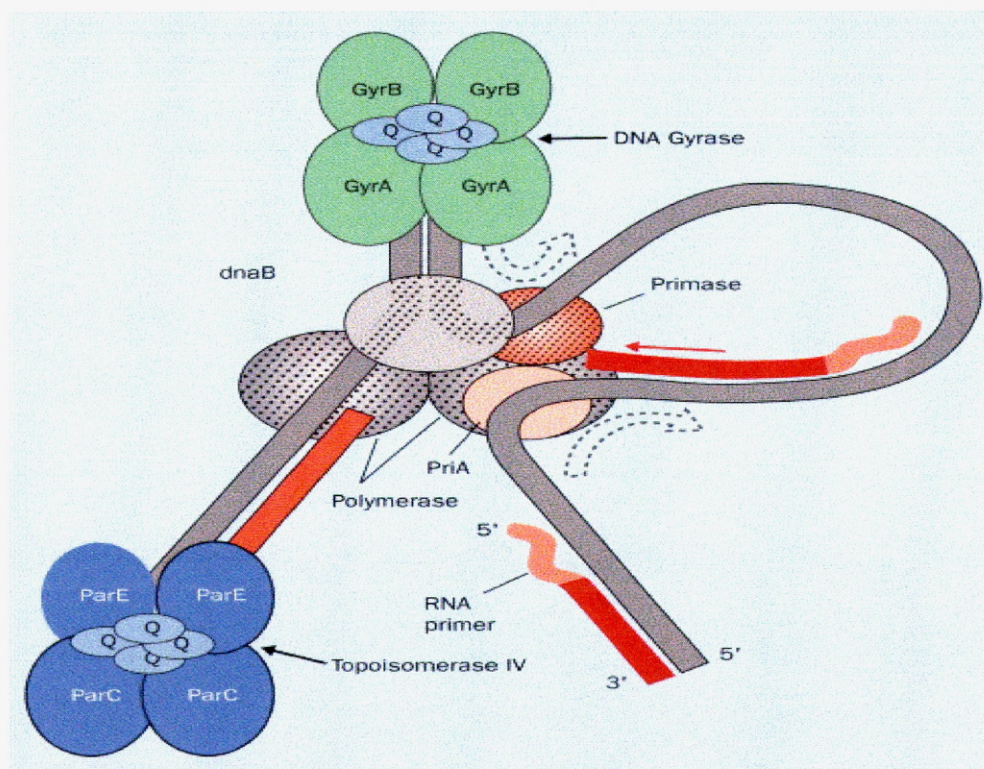


Figure 1.4: schematic diagram showing complex formation between fluoroquinolone antibiotics (Q) and topoisomerase IV and DNA gyrase. These complexes block the progression of the multicomponent DNA replication complex. GyrA and GyrB are subunits of DNA gyrase enzyme. ParC and ParE are subunits of topoisomerase IV enzyme. DnaB, primase, PriA and polymerase are components of the DNA replication complex (Hooper, 2002).

1.2.3 Roxithromycin

Roxithromycin (Figure 1.2C) is a semi-synthetic macrolide, structurally related to erythromycin. Unlike erythromycin, roxithromycin is acid stable, has fewer side effects and a wider spectrum of activity than erythromycin. It is active against Gram-positive and Gram-negative cocci, Gram-positive bacilli and some Gram-negative bacilli. Since it penetrates and accumulates within cells, it is active against intracellular pathogens like *Legionella*, *Chlamydia* and *Mycobacterium* spp (Bryskier, 1998; Sweetman, 2007). In addition, based on a suggested link between atherosclerosis and *Chlamydia pneumoniae* infection, roxithromycin and other macrolides are being investigated in the prevention of atherosclerotic cardiovascular diseases (Wiesli *et al.*, 2002; Grayston, 2003; Berman *et*

al., 2005). Roxithromycin is given orally in a dose of 150mg twice daily or 300mg once daily, which makes it more convenient to patients (Sweetman, 2007).

Macrolides act by inhibiting protein synthesis in bacteria. The exact mode of action of macrolides depends on the chemical structure of each compound, as the size of the molecule and the orientation of its chemical groups on the target binding sites determine its action (Gaynor and Mankin, 2005). So far three mechanisms of inhibiting protein synthesis have been identified for roxithromycin; (i) inhibition of the progression of long peptide chain synthesis by blocking the nascent polypeptide exit tunnel, a process that may cause (ii) stimulation of early peptidyl tRNA dissociation from the ribosome which results in incomplete peptide formation, (iii) prevention of 50S subunit formation or assembly in growing bacteria (Menninger and Otto, 1982; Champney and Burdine, 1996; Schlunzen *et al.*, 2001; Gaynor and Mankin, 2005).

Resistance to macrolides emerged in many countries shortly after their introduction to clinical use. In general, three mechanisms by which bacteria resist macrolides were described: the first mechanism is by modification of the target site. This modification could be a result of mutations in some of the ribosomal proteins. This mutation will result in the formation of a wider tunnel, through which the growing protein can pass through without being affected by the hindrance caused by the macrolides. The most predominant mechanism of target site modification is by dimethylation of single nucleotide in the 23S rRNA portion of the 50S subunit, by methyl transferase enzymes. This will result in reduced affinity of the site to macrolides and other antibiotics which are lincosamides and streptogramins type B. This pattern of resistance is known as MLS_B (macrolides-lincosamide-streptogramin B) resistance (Leclercq and Courvalin, 1991; Schmitz *et al.*, 2000; Gaynor and Mankin, 2005). The second mechanism has been described in some strains of staphylococci by which the bacteria produce esterase

enzymes, which hydrolyse macrolides rendering them inactive (Wondrack *et al.*, 1996). The third mechanism is by excreting the drug outside the cells via efflux pumps (Ross *et al.*, 1990).

1.3 *Staphylococcus aureus* virulence factors

The diversity of diseases caused by staphylococci is believed to be a consequence of virulence factors expressed by these microorganisms. *Staphylococcus* spp are known to express numerous virulence factors. These factors are either cell associated factors, such as capsular polysaccharide, bacterial components involved in biofilm formation and cell proteins used to adhere to host tissue, or secreted factors like proteins, enzymes and toxins (Figure 1.5). Some of the virulence factors expressed by staphylococci and a summary of their effects are shown in Table 1.1. Toxic shock syndrome toxin-1 and biofilm formation will be described in more detail.

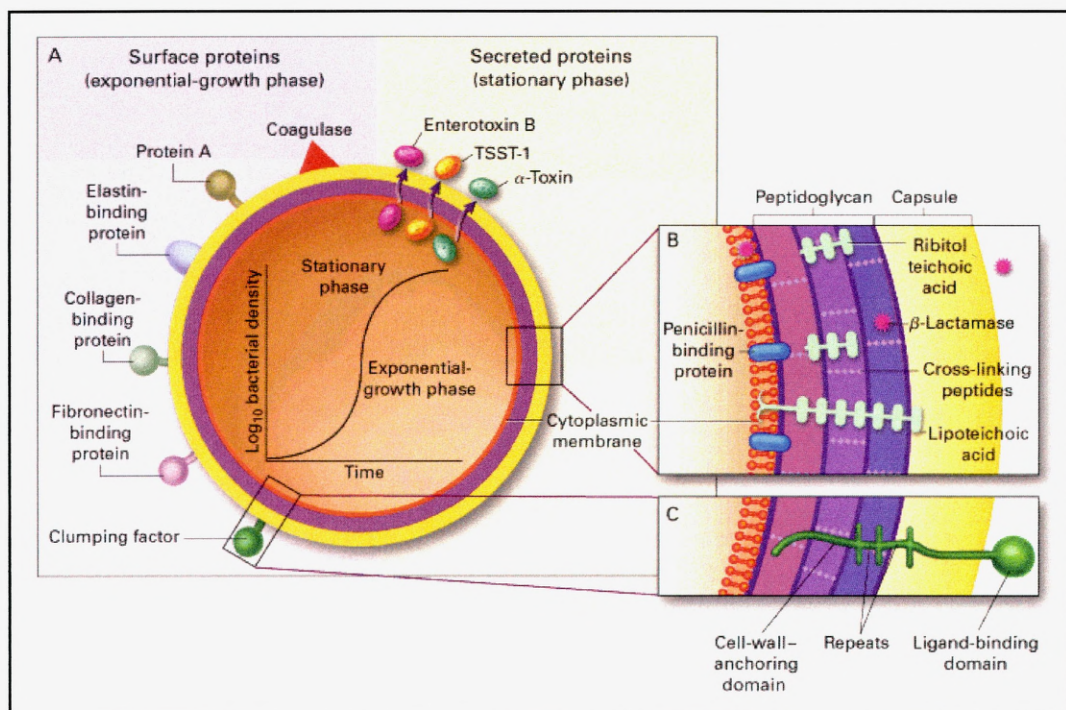


Figure 1.5: *Staphylococcus aureus* structure. Panel A shows cell associated proteins and secreted proteins; the synthesis of many proteins is dependent on the growth phase. Panels B and C show cross section of cell envelope (Lowy, 1998).

Chapter 1

Introduction

1.1 Staphylococcal infections and significance

Staphylococci are considered one of the most virulent human pathogens. In spite of the fact that they are common colonizers of human skin and mucous membranes, they are responsible for a wide spectrum of infections targeting a broad range of organs and tissues. Staphylococci can cause many forms of infections such as skin and soft tissue infections (including cellulitis, impetigo, furunculosis, scalded skin syndrome and soft tissue abscesses), deep-seated infections (endocarditis and osteomyelitis) and surgical site infections like wound infections and endophthalmitis (John, 2004; Casey *et al.*, 2007).

Staphylococcus aureus is a common cause of bacteraemia. Reports of *S. aureus* bacteraemia across England and Wales more than doubled from around 5000 in 1991 to more than 11,000 in 2000 (Figure 1.1) according to Communicable Disease Surveillance Centre (Anon, 2000). By releasing enterotoxins into food, *S. aureus* causes food poisoning (enterocolitis). In fact many outbreaks of food-borne illness are reported worldwide in which *Staphylococcus* spp were a common cause (John, 2004). Some strains also release superantigens, which interact with the immune system causing the serious systemic disease, Toxic Shock Syndrome.

Staphylococci are a major cause of nosocomial infections. *S. aureus* was the primary cause of nosocomial pneumonia and surgical site infections and the second leading cause of nosocomial blood stream infections (after coagulase negative staphylococci) in USA between 1990 and 1996 according to the National Nosocomial Surveillance System (Anon, 1996).

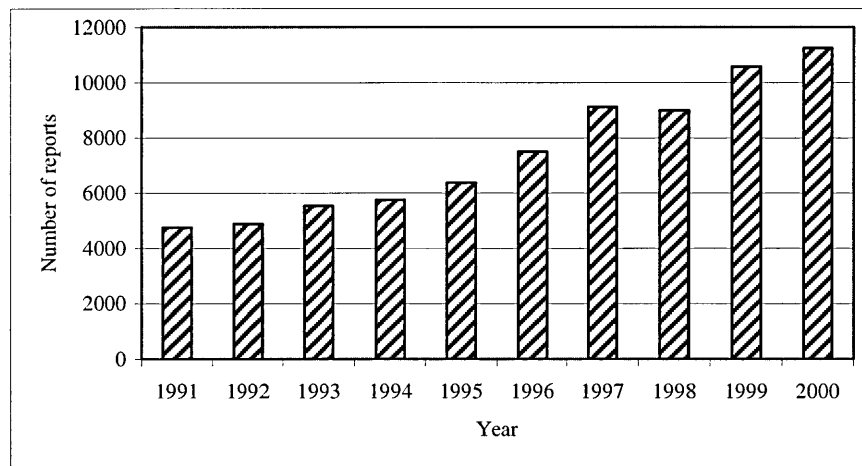


Figure 1.1: *Staphylococcus aureus* bacteraemia reports in England and Wales between 1991 and 2000 (Anon, 2000).

Clinically, it is well recognized that the most feared complication of implanted prosthetic devices is microbial infection; where *S. aureus* and *S. epidermidis* have a significant share (Bayston, 2000; Vuong and Otto, 2002). The problem with these infections is that most of them are incurable due to the formation of biofilms of bacteria, which are highly resistant to antimicrobials and their ultimate solution is surgical replacement of the infected device. The outcome of such operations is often catastrophic in terms of distress, trauma, possible disability of the patients and huge expenditure of national resources.

The importance of staphylococci has increased dramatically in the last two decades due to the increase in resistance to antimicrobials. In order to overcome penicillin resistant *S. aureus*, methicillin was developed. Unfortunately, strains resistant to methicillin emerged in hospitals shortly after its introduction. Later multi-resistant strains of methicillin-resistant *Staphylococcus aureus* (MRSA) prevailed, not only as nosocomial, but also as community acquired infections (Casey *et al.*, 2007).

Vancomycin was considered the drug of choice for MRSA infections, but increasing reports across the world of the occurrence of isolates with reduced susceptibility to vancomycin (vancomycin intermediate-resistant *Staphylococcus aureus*

(VISA)) (Hiramatsu, 1997) and later vancomycin resistant *S. aureus* (VRSA) (Siever, *et al.*, 2002) have alarmed the medical community and prompted the generation of new guidelines for prevention and control of staphylococcal infections in the health care system. Such resistance predicts a gloomy chemotherapeutic era in which efficient antibiotics against this organism may no longer be possible (Lowy, 2003; Casey *et al.*, 2007).

1.2 *Staphylococcus aureus* resistance to antimicrobials

Mechanisms of microbial resistance towards antibiotics can be categorised into three general groups; (i) direct inactivation of the drug, (ii) changing the organism's susceptibility to the antibiotic (for example by altering the target site) and (iii) reduction of the active molecule's concentration that reaches the target, either by preventing drug access into the target or by pumping the drug out of the organism (efflux pumps) (Piddock, 1999; Hogan and Kolter, 2002). In fact staphylococci employ all three mechanisms to resist antimicrobials.

In the following sections, the mechanism of action of selected antibiotics and staphylococcal resistance to these antibiotics is discussed in more details. The selected drugs were used in this project and chosen as representatives of different groups of antibiotics that have different modes of action within the bacterial cell.

1.2.1 Cefalexin

Cefalexin (Figure 1.2A) belongs to cephalosporin antibiotics, which are part of the β -lactam family. It is a first generation cephalosporin, administered orally and some of its derivatives administered parenterally. Cefalexin is active against Gram-positive cocci,

including penicillinase and non-penicillinase producing staphylococci and has moderate activity against Gram-negative bacilli (Sweetman, 2007).

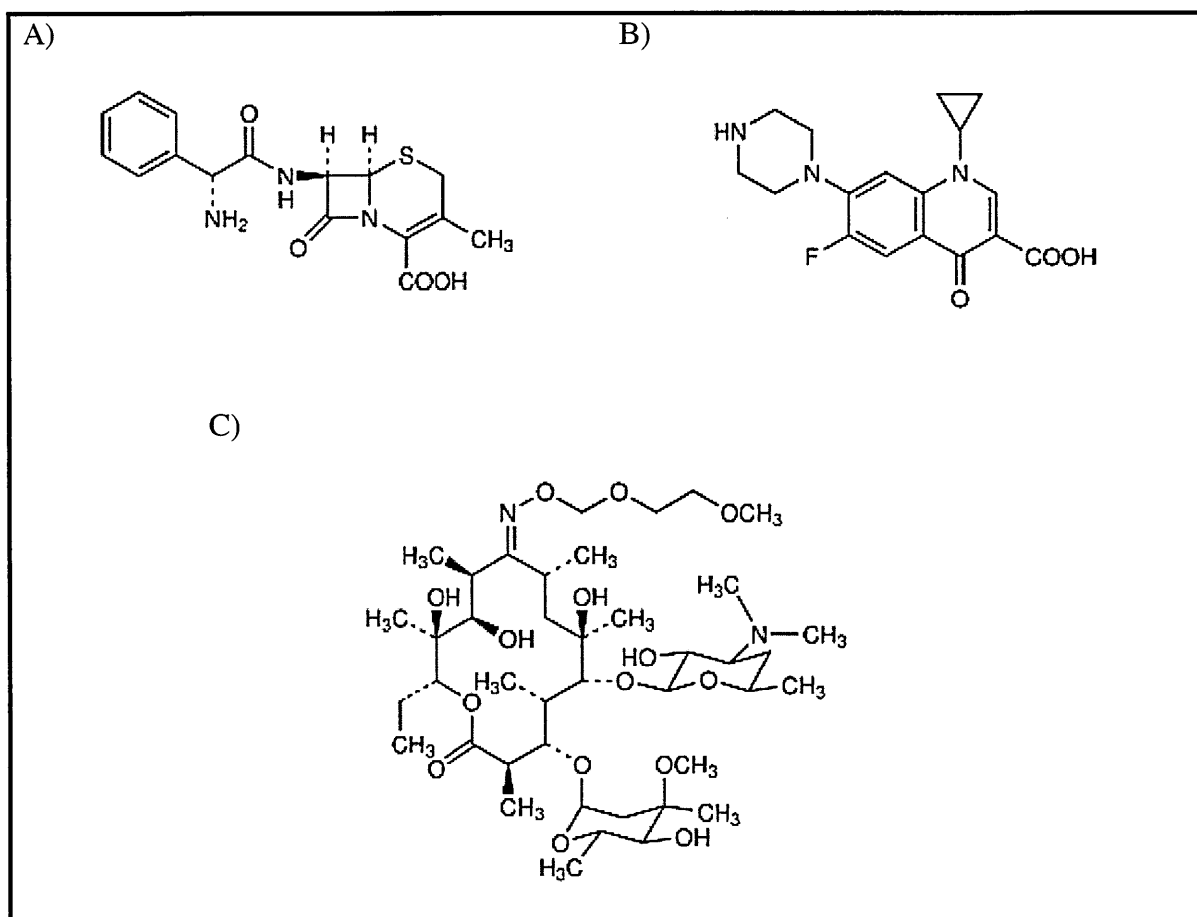


Figure 1.2: Chemical structure of (A) Cefalexin, (B) Ciprofloxacin and (C) Roxithromycin (adapted from Sweetman, 2007).

Cefalexin, and β -lactams in general, are bactericidal antibiotics that act through inhibition of cell wall synthesis in actively growing bacteria. These compounds are structurally similar to the terminal D-alanyl-D-alanine residue of the glycan strand of peptidoglycan and therefore, they are recognised as substrates by the enzymes responsible for transpeptidation and cross linking of peptidoglycan. Subsequently, a covalent bond is formed between the drug and the enzymes rendering the enzymes inactive (Figure 1.3). These enzymes belong to a group of enzymes called the Penicillin Binding Proteins

diminished production of biofilm associated protein (Bap), fibronectin binding protein and polysaccharide intercellular adhesin. These compounds are required in the first and second stage of biofilm formation and their reduction or loss would reduce biofilm formation dramatically (Beenken, *et al.*, 2003; Trotonda *et al.*, 2005).

1.5.3 Sigma factor B (σ^B)

Under normal conditions σ^B factor is bound to anti- σ^B factor preventing it from acting on RNA-polymerase enzyme. Upon stress conditions, such as extreme pH, osmolarity and temperature, σ^B is released and acts on more than 23 promoters. In a study, σ^B was found to down regulate the production of eleven exoproteins, among them enterotoxin B, α - and β -haemolysin and serine proteases (Ziebandt *et al.*, 2001), beside its role to repress *agr* system by inducing the synthesis of *agr* repressor molecule (Bischoff *et al.*, 2001).

The σ^B role in biofilm formation is not essential although it is important. Valle *et al.*, (2003) have shown that complete deletion of σ^B in a *S. aureus* clinical isolate did not affect polysaccharide intercellular adhesin (PIA) production or biofilm formation, while *sarA* deletion resulted in complete loss of ability to form biofilm. Interestingly, *sarA*- σ^B double mutation resulted in increased PIA production. One of the explanations suggested was the presence of a repressor to PIA synthesis that SarA inhibits its transcription while σ^B activates it. Recent study demonstrated that σ^B affects biofilm formation through enhancing phenotypic switch of *S. aureus* to biofilm positive phenotype (Valle *et al.*, 2007).

1.6 Hypothesis

The pathogenicity of *S. aureus* is due to its production of large number of virulence factors whether secreted or cell bound. Furthermore, the ability of bacteria to form biofilm is one of its virulence mechanisms. Virulence factors are regulated by quorum sensing systems, which mediate the environmental conditions (nutrients, pH, oxygen, presence of antibiotics) and the growth phase of the microorganism.

Numerous antimicrobial agents are used to treat bacterial infections. Drug doses are prescribed to achieve serum concentrations above the minimum inhibitory concentration (MIC) of the particular organism assuming that, achieving such concentration would be clinically effective. However, the exposure of bacteria to sub-inhibitory concentrations of antibiotics under clinical conditions is highly probable. For example, drug-drug interaction, drug-food interaction or certain clinical or health conditions of the patient, could result in decreased bioavailability of certain antibiotics into the systemic circulation and therefore, sub-therapeutic levels of the drug will be obtained (Deppermann and Lode, 1993; Cohn *et al.*, 1995; Komori *et al.*, 2007). On the other hand, establishing a certain drug concentration in serum does not necessarily guarantee achieving this concentration in tissues or site of infection (Ugurbas *et al.*, 2007), thus, bacteria invading tissues will be exposed to lower levels of antibiotics (Keren *et al.*, 1991). Moreover, systemic absorption of antibiotics applied topically will result in sub-therapeutic levels of these antibiotics (Thiboutot *et al.*, 2007) to possibly present asymptomatic infections or chronic bacteria colonizers in other parts of the body. Therefore, the effect of subinhibitory concentrations of antibiotics on microorganisms has developed much interest and research (Dunne, 1990; Rupp and Hamer, 1998; Rachid *et al.*, 2000; Winder, 2000; Winder *et al.*, 2000; Abdel Malek, 2002; Abdel Malek *et al.*, 2002; Al-Hmoud, 2002; Bernardo *et al.*, 2004; Masadeh, 2005; Koszczol *et al.*, 2006).

The main hypothesis forwarded in this thesis is that: exposing *S. aureus* to sub-MICs of antibiotics may have differing effects on its virulence expression. Additionally, antibiotic type, concentration and strain of bacteria are among the factors that will determine the net effect on the expression of certain virulence factor (Dickgiesser and Wallach, 1987; Gemmell and Ford, 2002; Edwards-Jones and Foster, 2002; Stevens *et al.*, 2007).

1.6.1 Aims of the study

It is the aim of this study to investigate the difference in expression of some virulence factors (coagulase, protease and Toxic shock syndrome Toxin-1 (TSST-1)) from *S. aureus* biofilms, when exposed to sub-MIC of three selected antibiotics (Cefalexin, Ciprofloxacin and Roxithromycin) and to investigate the ability of this pathogen to form biofilm when challenged with subinhibitory concentrations of these drugs. In addition, one of these virulence factors, TSST-1, will be isolated and its biological activity assessed based on the assumption that exposing biofilm cells to sub-MIC may have an impact at the transcription or translation level of this toxin resulting in a protein with altered activity.

625nm. The absorbance should be between 0.08 to 0.10 otherwise it should be adjusted by dilution. The suspension was distributed into screw capped tubes in 6mL aliquots and stored in a darkened cupboard at room temperature. Before use, the standards were vortexed vigorously to produce homogenous suspension (NCCLS, 2000).

2.8 Protein quantitation

In this project, two methods for protein quantitation were used: Bicinchoninic acid Assay (BCA) and modified Coomassie (Bradford) protein assay. BCA was used for quantitation of proteins at concentrations from 50 to 1000 $\mu\text{g mL}^{-1}$. Bradford microplate assay was used to quantitate proteins in the range of 1 to 25 $\mu\text{g mL}^{-1}$.

2.8.1 Bicinchoninic acid Assay (BCA) for protein quantitation

Bicinchoninic acid, sodium salt, is a stable, water-soluble compound that reacts with cuprous ions (Cu^{+1}) in alkaline media forming an intense purple complex. The concept of this assay relies on the reduction of cupric ions into cuprous ions by proteins. Two molecules of bicinchoninic acid then chelate cuprous ions forming intense purple complex that absorbs light at 562nm. The colour produced from this reaction is stable and is proportionally related to the increase of protein concentration over a broad range of concentrations. This method has good tolerance toward commonly encountered interferences like non ionic detergents and simple buffer salts. In addition, it has high sensitivity and low protein-to-protein variation (Smith *et al.*, 1985).

2.8.1.1 Preparation of BCA reagent

BCA reagent was prepared by mixing ready made Bicinchoninic acid solution (Sigma) with Copper II sulphate solution (Sigma) in 100:2 ratio immediately before use to produce a bright apple green coloured solution.

2.8.1.2 Preparation of standard calibration curve

A stock of bovine serum albumin (BSA) of 1mg mL^{-1} was prepared in distilled water. Aliquots were diluted in distilled water to obtain a concentration range of (0.05 to 1mg mL^{-1}). Aliquots ($50\mu\text{L}$) of each concentration were transferred to Eppendorf tubes, 1mL of BCA reagent was added, vortexed and incubated at 37°C for 30min. The absorbance was measured at 562nm using BCA reagent as a blank. The calibration curve was produced by plotting the absorbance of each sample against the corresponding protein concentration (Figure 2.4).

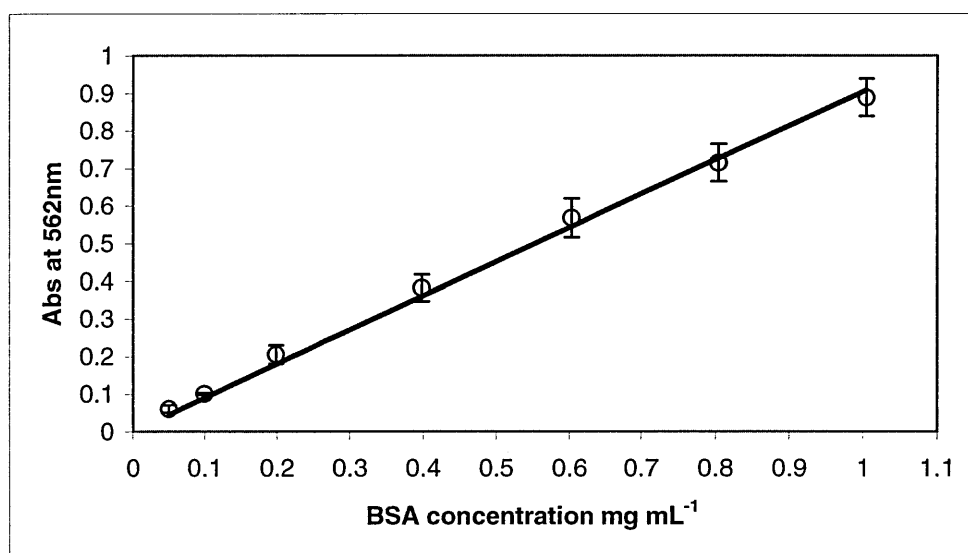


Figure 2.4: Standard calibration curve for bicinchoninic acid assay. Absorbance of standards at 562nm versus bovine serum albumin concentration (BSA). Each point represents average absorbance values. Error bars represent \pm standard deviation ($n=3$).

2.8.1.3 Analysis of samples

Aliquots (50 μ L) of samples were mixed with 1mL BCA reagent and incubated at 37°C for 30min. Their absorbencies were measured at 562nm using appropriate blanks and protein concentration was estimated from the calibration curve. Suitable blanks were prepared for each measurement, in which the blank solution contained all the components of sample solutions except the proteins.

2.8.2 Coomassie (Bradford) protein assay

Coomassie (Bradford) protein assay relies on the interaction of Coomassie brilliant blue G 250 dye with proteins (Bradford, 1976). Under acidic conditions, the dye is doubly protonated and in its most stable form. Acidic conditions turns the dye to brown colour with absorption maximum at 465nm. When it binds to proteins, an immediate shift in absorption maximum occurs from 465nm to 595nm accompanied by colour change to blue. The increase in absorbance at 595nm due to reaction with protein is monitored. At increasing protein concentrations, the colour response is non-linear, which necessitates the use of standard calibration curve with each set of samples. This assay is rapid, reproducible with low interferences from cations and carbohydrates. Some interference may occur with alkaline buffers or detergents but can be compensated by the use of suitable controls (Bradford, 1976).

2.8.2.1 Sample analysis using Coomassie protein assay kit (Pierce, USA)

The instructions provided by (Pierce, USA) for determining protein concentration using microplate protocol were followed. Bovine serum albumin standard solution 2000 μ g mL⁻¹ (Pierce, USA) was diluted in the same buffer as the sample to produce final BSA concentration range 2.5 to 25 μ g mL⁻¹. Aliquots (150 μ L) of each standard and

sample were added to 96 well-flat bottom microtiter plate in triplicates. Coomassie reagent solution (Pierce, USA) was mixed and equilibrated to room temperature. Immediately before use, Coomassie reagent solution was mixed gently and aliquots (150 μ L) were added to each well. A blank was prepared in triplicate by mixing 150 μ L reagent with 150 μ L buffer. The plate was shaken for 30seconds at medium speed and was incubated for 10 minutes at room temperature. The absorbance was measured at 595nm using ELISA reader (Rosys Anthos ht III, Anthos Labtecc Instruments, Salzburg, Austria). A calibration curve was plotted between the absorbance at 595nm against BSA concentration (Figure 2.5).

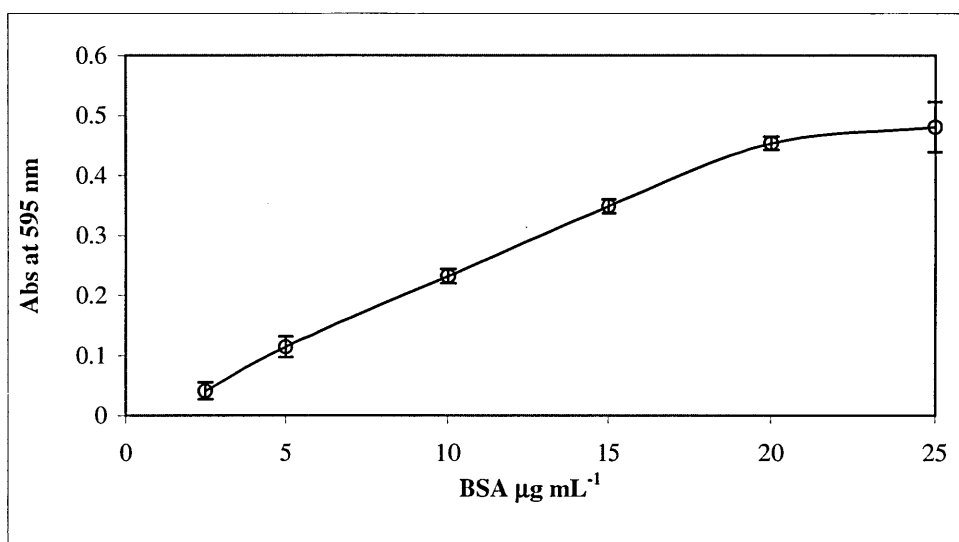


Figure 2.5: Standard calibration curve for microplate Bradford protein assay. Absorbance (Abs) at 595nm against bovine serum albumin (BSA) concentration. Each point represents average absorbance values after subtracting blank value. Error bars represent \pm standard deviation (n=3).

2.8.3 Preparation of cell pellets from biofilms and eluates and preparation of cell free eluate (eluate supernatants) from biofilms exposed to sub-MIC of antibiotics.

Biofilms of *S. aureus* NCTC 11962 were established on Sorbarod filters as was described earlier in this Chapter. Sorbarods were conditioned with 5mL normal saline before inoculation. Subsequently, the filters were inoculated with (3mL) of mid

exponential phase *S. aureus* suspension grown in brain heart infusion. The filters were incubated at 37°C. Sorbarods were perfused with BHI medium containing 1/16 MIC of one of the antibiotics (cefalexin, ciprofloxacin, roxithromycin) at a flow rate of 0.4mL min⁻¹ maintained by peristaltic pump. The amount of antibiotic required to prepare 1/16 MIC of each antibiotic, was taken from sterile stock solutions of antibiotics and was added to already sterilized and cooled BHI medium. After achieving the steady state after 48hrs, the filters were removed, sacrificed (adherent cells were removed) and resuspended in normal saline. Biofilm cells and eluate cells were pelleted by centrifugation using refrigerated centrifuge (Jouan CR312, Jouan Ltd, Ilkeston, UK) at 3500 rpm, 4°C, for 60 minutes. The supernatant of eluate cells was decanted and placed in centrifuge tubes for subsequent analysis. Pellets of biofilm and eluate cells were washed with distilled water three times (Berber *et al.*, 2003). If cell pellets were not used immediately they were stored at -20°C for further analysis.

2.8.4 Extraction of intracellular proteins from *S. aureus*

In order to extract intracellular proteins, the cells have to be lysed. Several procedures can be used to lyse cells such as physical, mechanical or chemical methods. In this study, mechanical disruption of cells by homogenization was used. In order to avoid excessive homogenization, the optimum time to produce lysis to cells was determined. 1.5mL of washed cell pellets were suspended in 3mL of normal saline (in subsequent work – see 4.3.3 and 4.4.1.1, protease inhibitors were included in the homogenisation buffer). The suspension was distributed in small equal aliquots into conical centrifuge tubes. The cell suspension of the first tube was homogenized by hand held homogenizer (Ultra Turrax T8, S8N-5G, IKA Labotechnic, Stauffer, Germany) for 1 minute on ice. The homogenizer was set at an arbitrary speed of 3, which was evaluated and used throughout

Chapter 2

General experimental methods

2.1 Introduction

The purpose of this chapter is to provide details of experimental techniques and reagents (composition and preparation) that are associated with testing protocols and analyses that are undertaken in subsequent chapters. In addition, identification and maintenance of cultures are described.

2.2 Reagents and media

Amersham Biosciences, Uppsala, Sweden (part of GE Health care)

Dithiothreitol (DTT); Dry strip cover fluid; Urea.

Fluka (a Sigma-Aldrich brand)

2-mercaptoethanol, N α -Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), N,N,N',N',Tetramethyl ethylenediamine (TEMED), Crystal violet; Polyethylene glycol (PEG 20000), Sodium dihydrogen phosphate monohydrate.

Oxoid LTD, Hampshire, England

Brain Heart Infusion (Code: CM0225); Brain Heart Infusion agar (Code: CM0375); Mannitol salt agar (Code: CM0085); agar bacteriological (agar No. 1, Code: LP0011); Tryptone Soya agar (Code: CM0131).

Riedel De Haen (a Sigma-Aldrich brand)

Congo red; Sodium chloride; Sodium phosphate dibasic heptahydrate; Trichloroacetic acid.

Sigma-Aldrich Company Ltd, Dorset, England

Amberlite weakly acidic cation exchanger, hydrogen form CG50; Ammonium persulfate; Azocasein; Bicinchoninic acid solution; Bromophenol blue sodium salt for electrophoresis; Copper II sulfate solution; Glycine for electrophoresis; Lectin from *Phaseolus vulgaris* (red kidney bean) phytohemagglutinin PHA-P, contains buffer salts and NaCl; Marker for 2D electrophoresis; Proteinase K (≥ 30 units/mg protein); Rabbit plasma citrated; Sodium carbonate anhydrous; Sodium lauryl sulfate; Sodium thiosulfate pentahydrate; Toxic Shock Syndrome Toxin 1 from *Staphylococcus aureus*; Trypan blue solution 0.4% prepared in 0.81% NaCl and 0.06% potassium phosphate dibasic.

The antibiotics used in the study

Cefalexin from Orchid Chemicals and Pharmaceuticals Ltd., India; ciprofloxacin HCl from Dr Reddy's Laboratories Ltd, India and roxithromycin from Alembic Ltd, India.

2.3 Maintenance of bacterial cultures

Staphylococcus aureus ATCC 6538, ATCC 27659, NCTC 11962 and *Staphylococcus epidermidis* ATCC 12228 were used in this project. The original cultures of these bacteria came from either American Type Culture Collection (ATCC) or The National Collection of Type Cultures (NCTC) (London, UK). Bacterial strains were chosen to have different characteristics from each others. While *S. aureus* ATCC 6538 strain is recommended for quality control purposes and for testing antimicrobial agents and disinfectants, ATCC 27659 is penicillinase positive strain and NCTC 11962 is Toxic Shock Syndrome Toxin 1 and enterotoxin A producing strain. *S. epidermidis* ATCC 12228 is recommended for antibiotic assays and quality control purposes. Cultures of these bacteria were maintained in tryptone soya agar (Oxoid) slopes in quadruplicate. One slope was used for inoculation for experimental purposes, the second was kept as a back

up slope in case of contamination. The third slope was used for further subculturing to renew the slopes and the forth was kept as a stock. The slopes were stored in a darkened cupboard at room temperature. They were replaced at monthly intervals. Each time culture identification and characterisation was performed.

2.4 Preparation and Sterilization of media

Several media were used in this project:

2.4.1 Brain heart infusion agar (BHA) (Oxoid, Code: CM0375) and Tryptone soya agar (TSA) (Oxoid, Code: CM0131).

These media were prepared according to manufacturer's instructions. They were sterilized by autoclaving at 121°C, 15psi for 15 minutes. When the media cooled down to about 50°C, they were poured in Petri dishes (9cm, Plastiques Gosselin, France) under laminar flow.

2.4.2 Brain Heart infusion (BHI) (Oxoid, Code: CM0375)

A highly nutritious media. It was prepared according to manufacturer's instructions and sterilized by autoclaving at 121°C, 15psi for 15 minutes.

2.4.3 Mannitol salt agar (Oxoid, Code: CM0085)

A high salt content medium (7.5% salt) that selects for staphylococci since most other bacteria are inhibited by the high salt content. The medium was prepared according to manufacturer's instructions and sterilized by autoclaving at 121°C, 15psi for 15 minutes.

2.4.4 Chemically defined medium (CDM)

CDM was prepared according to Dinning (1995). This medium is composed of four solutions (A, B, C and D); each one was sterilized individually and then mixed aseptically in a 1000:5:15:10 ratio respectively. Solution A was prepared by dissolving K_2HPO_4 (2.56g), KH_2PO_4 (2.08g) and NH_4Cl (1.00g) in 900mL distilled water. The pH was adjusted to 6.8 with either 1M HCl or 1M NaOH and the volume made up to 1L with distilled water. The solution was sterilized by autoclaving at 121°C, 15psi for 15 minutes. Solution B was prepared by adding ferric ammonium citrate (1.0g) and anhydrous calcium chloride (0.1g) to distilled water to make 100mL. The solution was sterilized by filtration (0.2µm pore size, sterile mixed cellulose ester filter, Advantec MFS, USA) by the aid of vacuum. Solution C was prepared by dissolving glycerol (46.45g) in 400mL water, pH adjusted to 6.0 if necessary, and then volume made up to 500mL by distilled water. It was sterilized by autoclaving at 121°C, 15psi for 15minutes. Finally, solution D was prepared by dissolving $MgSO_4 \cdot 7H_2O$ in 1L distilled water and then sterilized by autoclaving at 121°C, 15psi for 15 minutes.

2.5 Identification of bacteria

The following tests were performed to confirm the identity of *S. aureus* and *S. epidermidis* cultures obtained from ATCC and NCTC.

2.5.1 Catalase test

Catalase enzyme breaks down hydrogen peroxide (H_2O_2) into water and oxygen. It is produced by most cytochrome-containing aerobic and facultative bacteria except *Streptococcus* spp and *Micrococcus* spp. When catalase producing bacteria are exposed to hydrogen peroxide solution vigorous bubbling occurs (MacFaddin, 2000). In this test, one drop of freshly prepared hydrogen peroxide solution 6% (v/v) was placed on a glass slide.

A loopful of colonies grown on tryprone soya agar was transferred to a cover slip, which was placed over the drop of hydrogen peroxide. Vigorous bubbling indicates catalase positive bacteria (Anon, 2006).

2.5.2 Growth on selective medium-Mannitol salt agar

This medium contains mannitol and a pH indicator phenol red. Pathogenic staphylococci, like *S. aureus*, ferment mannitol producing acidic by-products, which turn the colour of the phenol red indicator yellow. Thus, coagulase positive staphylococci produce colonies with bright yellow zones, while coagulase negative staphylococci are surrounded by a red or purple zone (Figure 2.1).

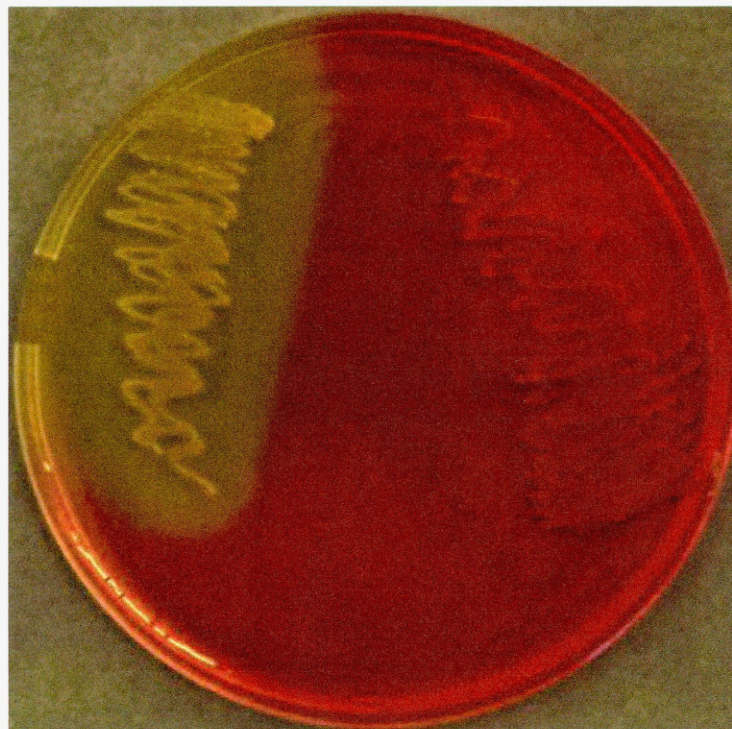


Figure 2.1: Mannitol salt agar medium. *Staphylococcus aureus* colonies grow surrounded by bright yellow zones (left of the plate), while *Staphylococcus epidermidis* colonies grow surrounded by red zones (right of the plate).

2.5.3 Staphylase test kit (Oxoid, Code: DR0595)

Staphylococci are divided into two groups by their ability to clot plasma, to coagulase-positive and coagulase-negative staphylococci. In human isolates, a coagulase positive result is almost exclusive to *S. aureus* (Mack *et al.*, 2006).

The Staphylase test is specific for *S. aureus* by detecting the presence of clumping factor on the cell surface. In this test, the presence of clumping factor is detected through clumping of fibrinogen-sensitised sheep red blood cells (Cremer and Gruneberg, 1988).

The test was performed according to manufacturer's instructions. Two bacterial smears were placed separately on a reaction card provided with the kit. Each smear was performed by transferring one to three colonies grown on tryptone soya agar plate. One drop of the test reagent containing rabbit fibrinogen-sensitised sheep red blood cells was added to the first smear and mixed with loop. One drop of the control reagent containing unsensitised sheep red blood cells was added to the second smear and mixed as negative control. *S. aureus* gives a positive result where clumping of the test suspension occurs during mixing. No clumping should occur with the negative control suspension. *S. epidermidis* gives a negative result with test suspensions.

2.5.4 Testing the ability of staphylococci to produce polysaccharide intercellular adhesin (PIA)

Three different formulations of Congo red agar have been described. These differ in the amount of carbohydrate supplement, agar and Congo red (Deighton *et al.*, 2001). In this study, Congo red agar was prepared according to Freeman *et al.*, (1989), by aseptically mixing 90mL of sterile liquid agar medium at 55°C, composed of (3.7g brain heart infusion broth, 5g sucrose and 1g agar bacteriological (agar No.1) in distilled water; sterilized by autoclaving) with 10mL sterile solution of Congo red (0.08% w/v; sterilized

by autoclaving). Congo red agar medium was poured in plates and left to solidify. A loopfull of bacterial culture was transferred from BHI agar slants and streaked on Congo red agar plate. The plates were incubated at 37°C over night. In this medium, PIA producers appear as black colonies with a dry crystalline consistency (Figure 2.2). PIA non producers remained pink.



Figure 2.2: Growth of *S. aureus* NCTC 11962 on Congo red agar plates. Polysaccharide intercellular adhesin producing strains appear as black colonies with a dry crystalline consistency.

2.6 Sorbarod Model as an *in vitro* model for growth of microbial biofilms

The Sorbarod model was developed by Hodgson *et al.*, (1995) for growth control of bacterial biofilms (Figure 2.3). The Sorbarod filter consists of a cylindrical paper sleeve, encasing a compacted concertina of cellulose fibres. A single Sorbarod filter (20mm x 10mm) was inserted into one end of 10cm polyvinyl chloride (PVC) tubing and

to the other end a 3mL disposable syringe was inserted. The plunger of the syringe was removed leaving the rubber seal within the syringe lumen. A disposable needle was inserted in the centre of the rubber seal. The whole assembly was sterilized by autoclaving. The medium was delivered by peristaltic pump (Gilson miniplus 3, France) to the assembly through sterile silicone tubings connected to the needle. The Sorbarod assembly was placed in an incubator at 37°C.

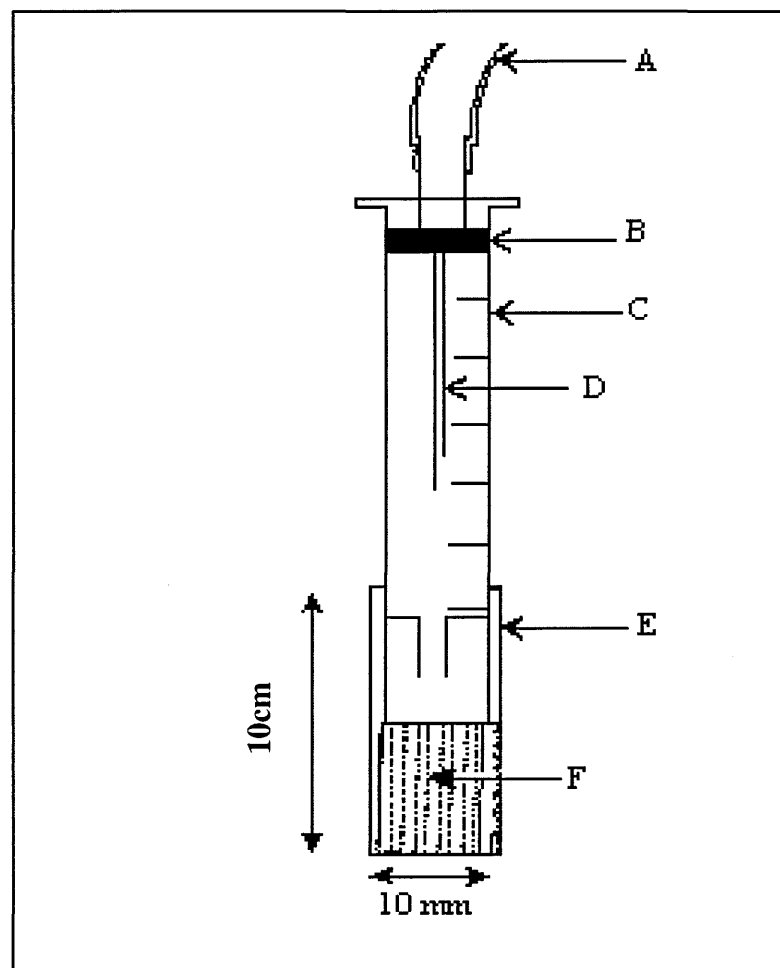


Figure 2.3: Diagram of the Sorbarod assembly showing the tubings delivering the medium (A); (B): the rubber plunger seal into which the needle (D) is inserted; (C) 3mL sterile disposable syringe; (E): 10cm PVC attached to the syringe from one end and the other end attached to Sorbarod plug (F), (Hodgson *et al.*, 1995).

Before pumping the media, Sorbarod filter was wetted with 5mL normal saline and then 3mL of mid exponential phase bacteria was added drop wise on to the top of the Sorbarod. Since the major portion of the inoculum attaches within 5 minutes (Deighton *et al.*, 2001) the flow was started 5 min after inoculation at a flow rate of 0.4mL min⁻¹. The effluent from Sorbarod (the eluate) was collected in sterile receivers.

2.6.1 Sacrifice of Sorbarod biofilm

After reaching the steady state, Sorbarod filters were removed from their PVC housing using sterile forceps. The paper sleeve surrounding the fibres was cut open with sterile scissors and the filter was placed into a sterile plastic disposable tube. The Sorbarod was then stored at -20°C for future use or was disintegrated for further analysis. In order to release biofilm cells, 9mL normal saline (0.9%w/v) was added to each Sorbarod filter and the filter was disintegrated by vortexing for 2 minutes. A tight fitting plunger was used to compress the diffusing fibres to the bottom of the tube, thus allowing the suspension of cells and matrix to be decanted. The Sorbarod was washed twice more. The decanted suspensions were collected in a sterile test tube. The cells were pelleted by centrifugation at 3500 rpm for 60 minutes (Jouan CR312, Jouan Ltd, Ilkeston, UK).

2.7 Turbidity standard for inoculum preparation

In order to standardize inoculum concentration or density, a barium sulfate turbidity standard was prepared equivalent to 0.5 McFarland. Bacterial suspensions adjusted to this standard would contain approximately 2×10^8 CFU mL⁻¹ for most species (NCCLS, 2000). The standard was prepared by slowly adding 0.5mL of BaCl₂ (1.175% w/v BaCl₂ H₂O) to 99.5mL of H₂SO₄ (1% v/v) with constant stirring. An aliquot of the suspension was placed in a quartz cuvette and measured spectrophotometrically at

Table 1.1: Some of the virulence factors (arranged alphabetically) expressed by Staphylococci and a summary of their effects.

Virulence factor	Description and Action	Reference
Broad specificity adhesin	These proteins bind to wide range of host proteins. The first described was Major histocompatibility complex type II analogue protein (Map), which binds to fibronectin, fibrinogen, vitronectin, bone sialoprotein and osteopontin. It has significant role in chronic staphylococcal infections and has inhibitory effect on T cells of the immune system. Others like extracellular adherence protein (Eap), which facilitates bacterial aggregation on eukaryotic cell substrates.	Lee <i>et al.</i> , 2002; Hussain <i>et al.</i> , 2002.
Capsular polysaccharide	The majority of clinical isolates produce capsules of serotype 5 or 8, which are repeating trisaccharide units. The capsule protects the bacterium from phagocytosis by impeding its interaction with leukocyte and plays an important role in inducing abscess formation.	Fournier <i>et al.</i> , 1984; Moreau <i>et al.</i> , 1990; Karakawa <i>et al.</i> , 1988; Thakker <i>et al.</i> , 1998; Tzianabos <i>et al.</i> , 2001.
Coagulase	An extracellular protein secreted by some species including <i>S. aureus</i> . It binds to prothrombin and activates fibrinolytic activity. Its role as virulence factor was shown in some staphylococcal infection models while excluded in others.	Moreillon <i>et al.</i> , 1995; Sawai <i>et al.</i> , 1997.
Collagen binding proteins	These proteins mediate adherence of the bacteria to collagen and cartilage. They are regarded as important virulence factors in septic arthritis, endocarditis and contact lens-associated ulcerative keratitis.	Patti <i>et al.</i> , 1994; Hienz, <i>et al.</i> , 1996; Rhem <i>et al.</i> , 2000).
Exfoliative toxins	A group of toxins that are involved in scalded skin syndrome disease. They have enzymatic activity capable of degenerating desmosomes required for cell-cell adhesion and causing intraepidermal skin peeling.	Amagai <i>et al.</i> , 2000.
Fibrinogen binding proteins (FbPs)	A group of proteins bound to the cell wall. They bind to fibrinogen specifically. These include clumping factor A (ClfA) and B (ClfB), which promote bacterial attachment to blood clots. ClfA plays significant role in inducing endocarditis in rat model. FbPs bind to platelets and inhibit their aggregation thus delaying wound healing.	Boden and Flock, 1989; Moreillon <i>et al.</i> , 1995; Shannon <i>et al.</i> , 2005.

Table 1.1 continued		
Fibronectin binding adhesins	Two related fibronectin binding proteins are expressed by most <i>S. aureus</i> strains. They mediate bacterial attachment to plasma clots and fibronectin which is a component of epithelial and endothelial surfaces. This attachment is considered a bridge required for tissue invasion. Also these proteins were shown to activate platelets, resulting in aggregation and thrombus formation, a step that is important for infective endocarditis.	Fowler <i>et al.</i> , 2000; Fitzgerald <i>et al.</i> , 2006.
Membrane active toxins	<p>These compounds act on the host cells' membranes causing lysis or cytotoxic effect. They include α-toxin (hemolysin), β-toxin, δ-toxin, leukocidin, Panton-Valentine leukocidin (PVL) and γ-toxin.</p> <p>α-toxin: produced by most <i>S. aureus</i> strains, it has haemolytic, cytotoxic (erythrocytes, mononuclear cells, epithelial and endothelial cells), dermonecrotic and neurotoxic effect. Some animals have high affinity binding sites on their erythrocytic membrane to the toxin, like rabbits, which make them 1000 times more susceptible to hemolysis than other animals with lower sensitivity like humans. α-toxin also induces release of proinflammatory cytokines and procoagulatory compounds. Strains expressing α-toxin were found to be more virulent than α-toxin mutants.</p> <p>β-toxin is a sphingomyelinase enzyme. Mostly expressed by staphylococci isolated from animals with mastitis. Its activity as haemolytic agent differs according to the animal species. It has leukotoxic effect.</p> <p>δ-toxin: it has a broad range of cytolytic activity with some surface activity properties. Rabbit erythrocytes are most sensitive to it while humans' cells are 1000 times less sensitive.</p> <p>PVL has leukotoxic effect and lytic effect on neutrophils and macrophages, which is species dependent.</p> <p>γ-toxin a haemolytic toxin with low leukotoxic activity, almost produced by all strains of <i>S. aureus</i>.</p>	Bhakdi and Tranum-Jensen, 1991; Dinges <i>et al.</i> , 2000.
Plasmin sensitive surface proteins	Expressed in some strains of <i>S. aureus</i> . It promotes cell binding to cellular lipids and glycolipids and promote bacterial cell-cell interactions.	Huesca <i>et al.</i> , 2002.
Polysaccharide intercellular adhesins	A linear polysaccharide. Considered the primary constituent of the exopolymeric matrix of <i>Staphylococcus</i> biofilm. It mediates biofilm formation and accumulation.	Cramton <i>et al.</i> , 1999; Mack <i>et al.</i> , 2004.

Table 1.1 continued		
Proteases	The major groups of these extracellular proteins are cysteine protease, metallo-protease and serine proteases. Produced by most strains. They contribute to bacterial invasion and spread through inactivating certain proteins in the immune system and structural proteins of the host. They also act on the proteins secreted by the pathogen itself like fibrinogen binding protein, and protein A, a role necessary to change bacterial status from adhesive to invasive.	Potempa <i>et al.</i> , 1988; Rapala-Kozik <i>et al.</i> , 1999; Shaw <i>et al.</i> , 2004.
Protein A	Cell wall anchored surface protein produced by most <i>S. aureus</i> isolates. Its role in virulence is controversial and depends on animal model used for the study. It binds to IgG molecules in the wrong way through Fc region, which principally will interfere with opsonization and phagocytosis. It binds to many mammalian proteins and to the Fab region of human immunoglobulin, which makes it a potential B cell superantigen. It also binds to von Willbrand factor (a glycoprotein produced in endothelium which mediates platelets aggregation to damaged endoepithelia) thus suggesting its role in endovascular infection.	Mascellino <i>et al.</i> , 1989; Jendeberg <i>et al.</i> , 1996; Hartleib <i>et al.</i> , 2000.
Staphylococcal pyrogenic toxin superantigens (PTSAGs)	Group of toxins that share similar biological characteristic. They include <i>Staphylococcus</i> enterotoxin (SE) A to Q excluding F and toxic shock syndrome toxin 1 (TSST-1). They induce pyrogenicity (fever), T-cell proliferation (superantigenicity), induction of cytokines and enhance lethality to endotoxins. SEs are responsible for food poisoning (gastroenteritis), they are potent emetics, heat stable and resist degradation in gastrointestinal tract while TSST-1 can pass mucous membranes.	Bohach <i>et al.</i> , 1990); Dinges <i>et al.</i> , 2000; Lee and Bohach, 2004.
Staphylokinase	An exoprotein enzyme. It activates plasminogen and causes thrombolysis and fibrinolysis, which help the bacteria in invasion and spread in the host tissues. Staphylokinase was found to induce secretion of α -Defensins peptides from neutrophils, interact with them and abolish their bactericidal activity.	Jin <i>et al.</i> , 2004; Braff <i>et al.</i> , 2007.

1.3.1 Toxic Shock Syndrome Toxin 1 (TSST-1)

1.3.1.1 The disease

Toxic shock syndrome (TSS) as a disease, was first described in 1978 in children as a systemic illness caused most probably by secreted toxins of *S. aureus*, since the

bacteria were isolated from localised tissues, but not from the blood (Todd *et al.*, 1978). This disease is caused by pyrogenic toxins released from some strains of *S. aureus* or streptococci (Bohach *et al.*, 1990). TSST-1 was the first toxin identified from this disease (Bergdoll *et al.*, 1981). In fact, not every strain producing the toxin would necessarily cause this disease. Several surveillance studies have isolated TSST-1 producing staphylococci from healthy carriers or asymptomatic persons and the nares were the most common site for this microorganism (Daghistani *et al.*, 2000; Warner and Onderdonk, 2004; Fueyo *et al.*, 2005). The Centres for Disease Control and Prevention in USA define toxic shock syndrome by five clinical criteria: fever, hypotension, rash, desquamation (unless the patient dies before desquamation) and abnormalities in three or more organ system (Hajjeh *et al.*, 1999).

In 1979 to 1980 toxic shock syndrome emerged as national health threat in USA, in women in the reproductive age group, with a high morbidity and mortality rate. Most of the cases occurred in menstruating women, and were linked to the use of high absorbency tampons (Shands *et al.*, 1980). The incidence of the disease in other countries, like Britain and Switzerland, was much lower than the States. This was attributed to the type of tampons used in these countries (De Saxe *et al.*, 1982; Vischer *et al.*, 1985). The link of the disease to menstruating women and tampon usage initiated a wide range of actions in order to increase the awareness of women and to change tampon usage and production. These actions led to dramatic decrease in TSS cases in the following years (Hajjeh *et al.*, 1999).

In general, Staphylococcal Toxic Shock Syndrome is categorized into two major types; menstrual and non-menstrual (Shands *et al.*, 1980; Reingold *et al.*, 1982). TSST-1 is responsible for almost 100% of menstrual TSS cases and only 50% of non-menstrual cases, of which the other 50% is caused mostly by staphylococcal enterotoxin B or C.

These non-menstrual cases may occur in any age group regardless of the patient gender (Schlievert, 2005).

A surveillance study between 1979 and 1996 in the United States found that during that period TSS menstrual cases were reduced significantly, while the proportion of non-menstrual cases (due to surgical wound infection in both genders, nonsurgical cutaneous lesions in children and the use of barrier contraceptives in women) has increased (Hajjeh *et al.*, 1999). Recent reports show a four fold increase in TSS in the Minneapolis-St. Paul (Twin Cities) metropolitan area between years 2000 and 2003 (Schlievert *et al.*, 2004). This increase was attributed partly to a strain of methicillin resistant *S. aureus*, termed USA 1100, which was able to produce ten to hundred times more TSST-1 *in vitro* than methicillin sensitive staphylococci (Schlievert, 2005).

1.3.1.2 TSST-1 as superantigen

TSST-1 belongs to staphylococcal pyrogenic toxin superantigens (PTSAgs). Being a superantigen, it exhibits highly potent non-specific lymphocyte stimulatory properties, causing 500 to 3000 times more stimulation to T-cells than in normal antigenic recognition reaction, which will lead to a severe immunological response (Dinges *et al.*, 2000; Schlievert, 2005). Normally, conventional antigen is degraded by antigen presenting cells (APCs) and presented by major histocompatibility complex class II molecules (MHC II), which are expressed on the surface of APCs to T-lymphocyte through a groove formed by α and β chains of T-cell receptor (TcR) (Figure 1.6). While superantigens are not degraded by APC, they bind intact to MHC molecules outside the conventional binding site and the complex formed by superantigen-MHC-APC, recognizes only the V β element of T cell receptor (Figure 1.6). Thus, any T-cell with appropriate V β element will be stimulated. As a result of this stimulation T-cells and

APCs will release enormous amounts of cytokines (interleukins, lymphokines and tumour necrosis factor). This reaction goes beyond the control of the regulatory system of the host leading to systemic shock and death (Dinges *et al.*, 2000; Lee and Bohach, 2004).

Superantigens have been shown to enhance the activity of endotoxins, which could be responsible for their lethal effect. This effect is believed to be a result of inhibiting reticuloendothelial system (liver) clearance of circulating endotoxins, which in turn will induce release of lethal amounts of monokines, mainly tumor necrosis factor- α (Schlievert, 1982; Fujiwaka *et al.*, 1986). TSST-1 also causes hypotension and shock, which could be attributed to its interaction with endothelial cells (Dinges *et al.*, 2000). In a study, TSST-1 was shown to have a cytotoxic effect on porcine aortic endothelial cells at high concentration, while at lower concentrations it caused leakage of albumin from these cells (Lee *et al.*, 1991).

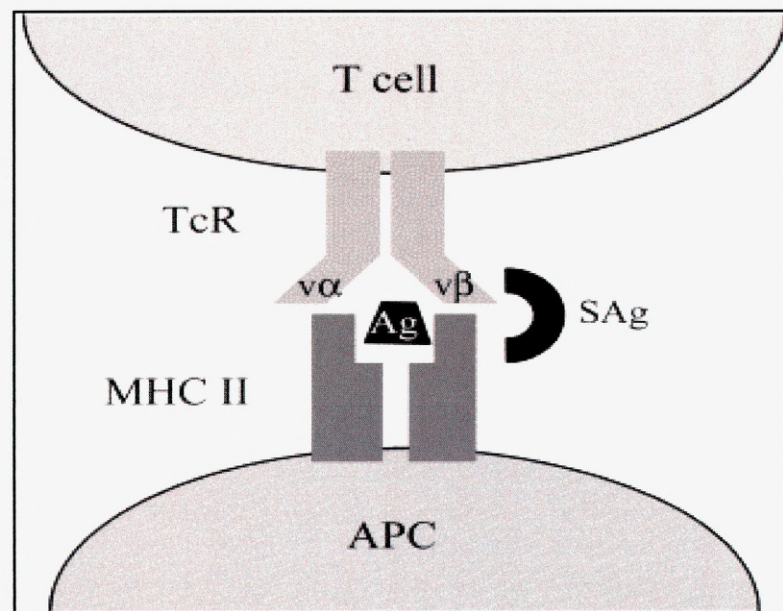


Figure 1.6: Stimulation of T cell by a conventional antigen (Ag) or by a superantigenic toxin (SAg). TcR: T cell receptor; APC: antigen-presenting cell; MHC II: major histocompatibility class II complex molecule (Muller-Allouf *et al.*, 2001).

1.3.1.3 TSST-1 structure and activity

Toxic shock syndrome toxin 1 is encoded by *tst* on the staphylococcal chromosome in a mobile genetic element called a pathogenicity island (Lindsay *et al.*, 1998). The gene is translated into a 234 amino acid precursor protein (Figure 1.7) with a molecular weight of 26.3kDa and isoelectric point of 8.8 (Blomster-Hautamaa *et al.*, 1986; <http://www.expasy.org>). This precursor has an N-terminal signal peptide composed of 40 aminoacids that is required in order to transfer the newly synthesized protein from the ribosome to the translocation machinery in the cytoplasmic membrane. Before secretion, the signal protein is cleaved from the Alanine/Serine point (aminoacids 40/41) to give the mature protein (Blomster-Hautamaa, *et al.*, 1986; Dinges, *et al.*, 2000; Sibbald, *et al.*, 2006). The mature toxin is a single polypeptide chain composed of 194 amino acids (Figure 1.7) with molecular weight of 22 kDa and isoelectric point of 7.2 (Blomster-Hautamaa *et al.*, 1986). Structurally, TSST-1 is composed of two adjacent domains (Figure 1.8). Domain A contains long central α -helix and a short N-terminal helix. The central helix is surrounded by five strand β -sheets. Domain B is composed of a barrel motif made from five β -strands (Prasad *et al.*, 1993). Studies on TSST-1 crystal forms revealed the presence of a zinc ion between two symmetrical molecules. Zinc was found to potentiate mitogenic activity at micromolar concentrations, but is not essential for activity (Prasad *et al.*, 1997). Studies on TSST-1 mutants revealed that major histocompatibility complex II (MHC II) contact residues are localized within the β -barrel, whereas superantigenic activity is localized to the central α -helix. In fact, the superantigenic activity of TSST-1 and the binding properties to MHC have been shown to be separable (Wahlsten and Ramakrishnan., 1998).

Single or double aminoacid mutations in the β -barrel abolished the ability of TSST-1 to bind to MHC II, while mutations to the α -helix resulted in significant

reduction in superantigenic activity and lethality (i.e. inducing lethal toxic shock syndrome). On the other hand, some mutations in the α -helix resulted in more superantigenic toxin, but with lower lethality effect, suggesting that lethality is not necessarily connected to T-cell proliferation and can be related to cytokine release, direct interaction with endothelial cells or endotoxin enhancement. However, the antigenicity of TSST-1, which is measured by reactivity of the toxin to its specific antibody in some TSST-1 mutants, was not affected (Blanco *et al.*, 1990; Murray *et al.*, 1996).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1	Met	Asn	Lys	Lys	Leu	Leu	Met	Asn	Phe	Phe	Ile	Val	Ser	Pro	Leu	15
16	Leu	Leu	Ala	Thr	Thr	Ala	Thr	Asp	Phe	Thr	Pro	Val	Pro	Leu	Ser	30
31	Ser	Asn	Gln	Ile	Ile	Lys	Thr	Ala	Lys	Ala	Ser	Thr	Asn	Asp	Asn	45
46	Ile	Lys	Asp	Leu	Leu	Asp	Trp	Tyr	Ser	Ser	Gly	Ser	Asp	Thr	Phe	60
61	Thr	Asn	Ser	Glu	Val	Leu	Asp	Asn	Ser	Leu	Gly	Ser	Met	Arg	Ile	75
76	Lys	Asn	Thr	Asp	Gly	Ser	Ile	Ser	Leu	Ile	Ile	Phe	Pro	Ser	Pro	90
91	Tyr	Tyr	Ser	Pro	Ala	Phe	Thr	Lys	Gly	Glu	Lys	Val	Asp	Leu	Asn	105
106	Thr	Lys	Arg	Thr	Lys	Lys	Ser	Gln	His	Thr	Ser	Glu	Gly	Thr	Tyr	120
121	Ile	His	Phe	Gln	Ile	Ser	Gly	Val	Thr	Asn	Thr	Glu	Lys	Leu	Pro	135
136	Thr	Pro	Ile	Glu	Leu	Pro	Leu	Lys	Val	Lys	Val	His	Gly	Lys	Asp	150
151	Ser	Pro	Leu	Lys	Tyr	Gly	Pro	Lys	Phe	Asp	Lys	Lys	Gln	Leu	Ala	165
166	Ile	Ser	Thr	Leu	Asp	Phe	Glu	Ile	Arg	His	Gln	Leu	Thr	Gln	Ile	180
181	His	Gly	Leu	Tyr	Arg	Ser	Ser	Asp	Lys	Thr	Gly	Gly	Tyr	Trp	Lys	195
196	Ile	Thr	Met	Asn	Asp	Gly	Ser	Thr	Tyr	Gln	Ser	Asp	Leu	Ser	Lys	210
211	Lys	Phe	Glu	Tyr	Asn	Thr	Glu	Lys	Pro	Pro	Ile	Asn	Ile	Asp	Glu	225
226	Ile	Lys	Thr	Ile	Glu	Ala	Glu	Ile	Asn							

Figure 1.7: Amino acid sequence of TSST-1 precursor showing the signal peptide (amino acid 1-40) and the mature TSST-1 sequence (amino acid 41-234). (<http://www.expasy.org/cgi-bin/sprot-ft-details.pl?P06886@CHAIN@41@234>). (Accessed on 2/09/2007).

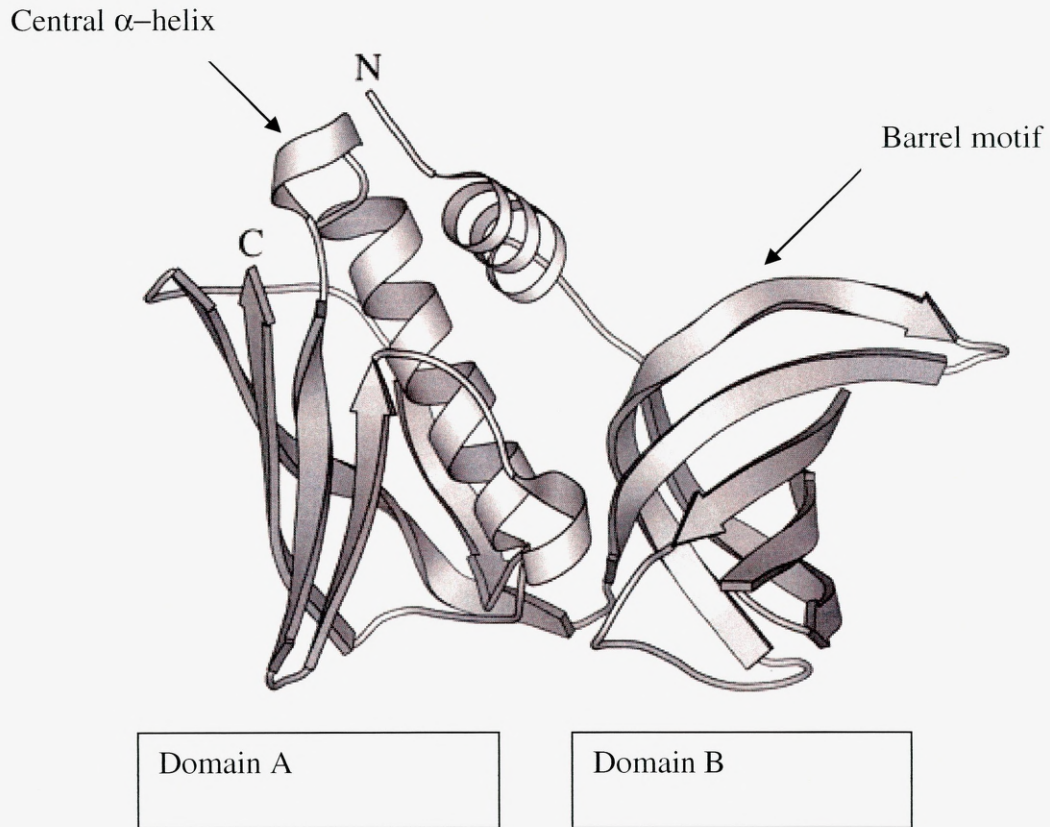


Figure 1.8: Ribbon Diagram of TSST-1 showing the N and C terminus of the protein. The central α -helix and the β -barrel motifs are indicated by arrows (adapted from Dinges *et al.*, 2000).

1.4 Biofilm formation

The ability of bacteria to form biofilms is considered one of the most important virulence effects of concern in the medical community, as these structures are highly resistant to antimicrobials and to the host defences. Biofilms can form on inert surfaces like catheters, prosthetic devices and contact lenses, on dead tissues like bone sequestra or even on living tissues (Costerton *et al.*, 1999).

Staphylococci have been shown to represent a large proportion of isolates from infected prosthetic devices. Most of the prosthetic device infections occur at the time of surgical placement of the device. Since staphylococci colonize nasal passages and the skin, they can gain access via medical staff or the patient to the device (Kluytmans *et al.*,

1997; Bayston, 2000). In recent surveys, biofilms of staphylococci were found to be the main causative reason of endocarditis according to survey carried out in 25 European countries (Tornos *et al.*, 2005). These biofilms were also found to be involved in other diseases like otitis media, bacterial prostatitis and cystic fibrosis (Donlan and Costerton, 2002).

Staphylococcus spp biofilm formation passes through three major phases (Higashi and Sullam, 2006). The initial phase is the adherence of the organism to a surface, where forces like hydrophobic, ionic and van der Waals interactions mediate cell adherence to inert surfaces. On the other hand, interaction with biotic surfaces or with tissues occurs through specific molecular adhesins. In the case of staphylococci these adhesins could be fibronectin binding protein, fibrinogen binding protein, protein A or clumping factors. A surface protein called biofilm associated protein (Bap) was found to play a role in biofilm formation by affecting the first stage and to some extent the second stage. This protein promotes non-specific interaction through enhancing surface hydrophobicity of bacteria. The ability of *bap* mutants to attach to polystyrene was significantly reduced and they were not able to form biofilm (Cucarella *et al.*, 2001). Other physicochemical factors such as oxygen tension, pH, hydrodynamic flow and nutrient conditions also affect this phase. The second phase involves cell division and the formation of an exopolysaccharide matrix that aggregates the cells into multilayers. In this phase, *S. aureus* and *S. epidermidis* produce exopolysaccharide matrix to facilitate cellular aggregation. Several studies have shown that this material is composed of polysaccharide intercellular adhesin (PIA, also known as slime or polymeric N-acetyl glucosamine) (Joyce *et al.*, 2003; Mack *et al.*, 2004; Higashi and Sullam, 2006). PIA expression was found to be induced by anaerobic conditions and such conditions may be encountered clinically in some diseases like airway plugs of patients with cystic fibrosis (Cramton *et al.*, 2001).

In *S. aureus*, α -toxin has been shown to be an essential compound for cell-to-cell interaction, but not for attachment to surfaces. This cellular interaction is required for multilayer formation in the second phase of biofilm formation (Caiazza and O'Toole, 2003). Bap protein in this stage has conflicting roles as its presence in certain infection models was necessary for biofilm formation (Cucarella *et al.*, 2004), while in others, its absence has no effect (Cucarella *et al.*, 2001). However, there is evidence that Bap has a biofilm potentiating relationship with PIA (Higashi and Sullam, 2006) or even a compensatory role if PIA is deficient (Cucarella *et al.*, 2004). However, *in vitro* studies have shown that *bap* mutants have weak intercellular interactions (Cucarella *et al.*, 2001). The final phase is the spatial arrangement of encased bacteria into a complex three dimensional structure containing channels through which nutrients and waste products circulate (Dunne, 2002; Higashi and Sullam, 2006). This phase is regulated and controlled by quorum sensing systems (Davies *et al.*, 1998; Maddula *et al.*, 2006).

The use of antibiotics to treat such infections has proved to be ineffective in most of the cases. Antibiotics may reverse the symptoms caused by planktonic cells released from the biofilm, but fail to eradicate the biofilm, especially if the infection is due to *S. aureus* or *Candida* (Darouiche, 2004). For this reason, biofilm infections of prosthetic devices cause recurring symptoms with antibiotic therapy and the ultimate solution is the surgical removal of the device (Table 1.2) (Costerton *et al.*, 1999).

Table 1.2: Clinical and economical consequences of biofilm infections of implanted devices in the United States (adapted from Darouiche, 2004).				
Implant	Implants Inserted in the U.S. Annually No.	Projected Infections of Implants Annually No.	Average Rate of Infection %	Estimated Average Cost of Combined Medical and Surgical Treatment (U.S. \$)
Cardiovascular :				
Mechanical heart valve	85000	3400	4	50000
Vascular graft	450000	16000	4	40000
Pacemaker–defibrillator	300000	12000	4	35000
Ventricular assist device	700	280	40	50000
Orthopaedic :				
Joint prosthesis	600000	12000	2	30000
Fracture-fixation device	2000000	100000	5	15000
Neurosurgical — ventricular shunt	40000	2400	6	50000
Plastic — mammary implant (pair)	130000	2600	2	20000
Urologic — inflatable penile implant	15000	450	3	35000

There are many possible mechanisms that account for biofilm resistance to antimicrobials, which vary with the microorganism forming the biofilm, the drug applied and the environment. No one mechanism can explain the increased resistance to antimicrobials. Some of the characterized mechanisms include:

1. Restricted penetration of antibiotics into biofilm.

It has been shown that biofilms retard the diffusion of some antimicrobials and large molecules of the host defence system. This retarded diffusion will decrease the concentration of antimicrobial entering the biofilm, allowing enzymes like β -lactamase to inactivate the incoming antibiotic (Lewis, 2001).

Souli and Giamarellou (1998) showed that the diffusion of one of the quinolones, pefloxacin, and some glycopeptides (vancomycin, teicoplanin, but not daptomycin) was retarded by staphylococcal slime. In another experiment, tobramycin penetration through *Pseudomonas aeruginosa* biofilms was retarded for 12 hours (Walters *et al.*, 2003).

2. Decreased growth rate.

Slow growth of the bacteria is observed in mature biofilms. Since some antibiotics act on growing cells, this could explain the resistance of some microbial biofilms to certain antibiotics. For example, penicillin and ampicillin do not kill non-growing cells, and it was found that their activity is proportional to growth rate (Lewis, 2001). Duguid *et al.*, (1992) proved that susceptibility of *S. epidermidis* biofilms towards tobramycin increased significantly with increasing growth rate. Eng *et al.* (1991), by controlling growth rate of bacteria through nutrient limitation, were able to demonstrate that stationary phase Gram-negative organisms were resistant to a wide range of antibiotics, where only fluoroquinolone antibiotics produced bactericidal effects. Furthermore, no class of antimicrobial agent in that study was bactericidal in the range of 3 orders of magnitude killing versus growth limited *S. aureus* (Eng *et al.*, 1991).

3. Expression of specific genes that contribute to biofilm cell resistance:

Genetic studies revealed that generally more genes were expressed in biofilms than in batch culture of planktonic cells (Jiang and Pace, 2006). Several studies indicated that high cell density induces stress response in the bacteria with the resulting expression of stress response factors that protect the organism from

heat/cold shock, pH variation and chemical agents (Brooun *et al.*, 2000; Mah and O'Toole, 2001). Studies in *Escherichia coli* suggested that growth within biofilm initiates stress response with production of trehalose (an osmo-protectant) and catalase (Liu *et al.*, 2000).

4. High rate of gene transfer.

Due to the close contact of bacteria and their spatial stability in biofilms structure, genes transfer by conjugation was found to be much higher than between planktonic cells suspensions and this is believed to increase the spread of antibiotic resistance genes (Jiang and Pace, 2006).

5. Biofilm microenvironment.

The microenvironment might have a role in tolerance of biofilms to antibiotics. It has been suggested that oxygen limitation in a 48 hours old *P. aeruginosa* biofilm, explains 70% of the protection afforded to biofilm cells against tobramycin, ciprofloxacin, carbencillin, ceftazidime and chloramphenicol (Borriello *et al.*, 2004; Walters *et al.*, 2003).

6. Heterogeneity within a biofilm.

Any given cell within the biofilm will experience a different microenvironment compared with other cells in the biofilm in terms of gradients of nutrients, waste products and signaling factors. This heterogeneity will result in the establishment of gradients in response to antimicrobials depending on the location of the cell. Fleroxacin caused extreme elongation to *Pseudomonas*

biofilm cells located close to the exposed side of the biofilm compared to other cells (Mah and O'Toole, 2001).

7. Persister cells.

It has been observed that when exposing *Pseudomonas* spp biofilm cells to antibiotics, most of the cells were killed at concentrations of antibiotics not much higher than those of planktonic cells, except a very small fraction of cells which tolerated high concentrations of drugs. These cells are known as persister cells (Brooun *et al.*, 2000). Persister cells are not mutants; rather, they are variants that are susceptible to growth inhibition as other biofilm cells, but are resistant to killing. Persister variants are believed to be responsible for the high resistance of biofilms and to play a role in recurrence of biofilm infection when the treatment is stopped (Lewis, 2001; Jiang and Pace, 2006).

1.5 Gene regulation of virulence factors

The ability of staphylococci to produce a wide variety of virulence factors, which are expressed during different stages of cell cycle or infection, assumes that the expression of virulence genes is regulated and coordinated in response to environmental signals. For instance, the initial stages of colonization require the bacteria to produce cell-surface molecules to aid the pathogen in adhesion to surfaces, while at a later stage, the bacteria release toxins and enzymes to enhance their spread and invasion. The way the bacteria control these numerous processes suggests the existence of global regulators (Higashi and Sullam, 2006).

S. aureus generally has three types of global regulators: the first one is the two component signal transduction systems (TCSs), which is typically composed of

membrane-associated proteins that extend through cytoplasmic membrane. These proteins bind to factors from the environment and initiates intracellular responses resulting in changes in gene expressions. The *agr* (accessory gene regulator) locus is the most studied TCS global regulator. The second global regulator is a group of transcription factors that mediate gene expression by binding to promoter regions of other transcription factor genes. SarA was the first one identified in this group. The third type is sigma factor B (σ^B), which is a transcription factor that induces transcription of genes needed to respond to stress conditions (Arvidson, 2004). However, some of the virulence factors themselves could have regulatory effects. TSST-1 and enterotoxin B were shown to act as global repressors to exotoxin synthesis, an effect which was attributed to the precursor of these toxins rather than the mature protein (Vojtov *et al.*, 2002). In addition, proteases were found to cause down regulation to cell-associated proteins like protein A and fibronectin binding protein (Karlsson *et al.*, 2001). Interestingly, some of the N-acyl homoserine lactones, the quorum sensing mediators of many Gram-negative bacteria, have been shown to inhibit *S. aureus* growth in a concentration-dependent-manner, while at subgrowth-inhibitory concentration they down regulate exotoxin production and *sarA* and *agr* expression, which are the quorum sensing system of *S. aureus* (Qazi *et al.*, 2006).

1.5.1 The accessory gene regulator (*agr*)

The *agr* locus is the major quorum sensing system of staphylococci. It is responsible for coordinating the switch of protein expression *in vitro* in *S. aureus* from surface proteins to exoproteins in post exponential phase (Arvidson, 2004). It has been shown to up regulate more than 100 genes and down regulate 34. About 80% of these genes were related to cellular processes and 20% responsible for encoding virulence factors (Dunman *et al.*, 2001). The effect of *agr* system on biofilm formation seems not to

be significant, as mutations of the *agr* system do not affect *S. aureus* biofilm formation (Beenken *et al.*, 2003). However, other studies found that disruption of the *agr* system could either enhance, inhibit, or even have no effect on biofilm formation. These effects depend on the environment and experimental conditions (Yarwood *et al.*, 2004).

The *agr* locus consists of two divergent transcriptional units, RNAII and RNAIII which are transcribed from promoters P2 and P3 respectively (Arvidson, 2004). RNAII codes for four polypeptides AgrB, AgrD, AgrC and AgrA, which activate transcription from the *agr* promoters, while RNAIII is the actual effector of virulence gene regulation (Higashi and Sullam, 2006). AgrD is the peptide precursor of autoinducing peptide (AIP), which is processed by AgrB (a membrane protease) to a mature form (Figure 1.9). AIP binds to AgrC (the *agr* system receptor) and causes its activation. The activated AgrC in turn activates AgrA (the response regulator), which stimulates the transcription of P3, resulting in encoding regulatory RNA molecule (RNAIII) that acts as primary effector of *S. aureus* quorum sensing. In addition, AgrA induces expression of Agr proteins through RNAII transcription by stimulating the transcription of P2 (Figure 1.9) (Arvidson, 2004; Horswill *et al.*, 2007). The transcription of RNAIII occurs in mid to late exponential phase resulting in the stimulation of expression of extracellular proteins, toxins and enzymes, but repressing surface protein genes (Novick *et al.*, 1993). It has been shown that each AIP activates only the *agr* system of strains of its own group and may act as inhibitor between different strains (Lyon *et al.*, 2002).

the study. In order to remove cell debris from the solution (cytosol), the homogenized suspension was centrifuged at 13000 rpm (Genfuge 24D, Progen, Mexborough, UK) for 5 minutes. The absorbance of the supernatant was measured at 260nm using spectrophotometer (Genesys 10UV, Thermo Spectronic, New York, USA). Normal saline was used as a blank. The second tube of cell suspension was homogenized for two minutes and the absorbance of the supernatant at 260nm was recorded. The same procedure of homogenization and subsequent measuring of absorbance was continued for the other tubes of cell suspensions, where each tube was homogenized for an extra one minute more than the previous one, until no increase in absorbance values was noticeable. By plotting the absorbance at 260nm against homogenization time, the minimum time required for homogenization can be determined. Figure 2.6 shows that the optimal homogenization time is 5 minutes at the set speed (speed 3).

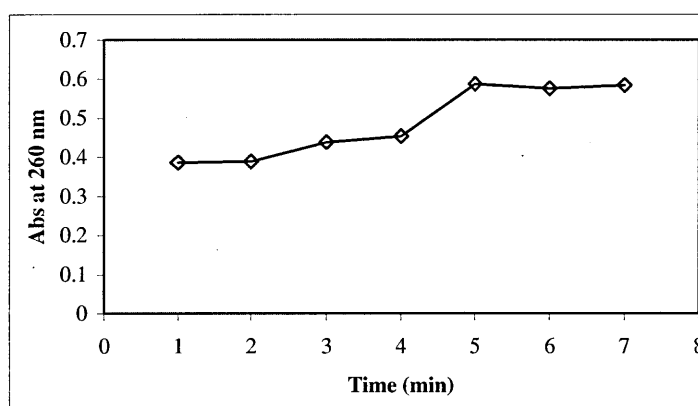


Figure 2.6: Determination of optimal homogenization time of *S. aureus* using hand held homogenizer at speed 3. Absorbance at 260nm vs homogenization time.

2.8.5 Extraction of exoproteins from eluate supernatant

A portion of the eluate supernatant (45mL) was placed in a centrifuge tube and placed on ice. Trichloroacetic acid (TCA) (5g) was dissolved in the eluate supernatant to give a final concentration of 10% w/v. The solution was left on ice for 30 min to allow for protein precipitation. In order to isolate the precipitated proteins, the tube was centrifuged

at 4°C (Jouan CR312, Jouan Ltd, Ilkeston, UK) at 3500 rpm, for 1 hr. the supernatant was decanted and discarded. The precipitated proteins were washed five times by ice-cold ethanol (99%) to remove the residual TCA. The washed proteins were dried by nitrogen gas to prevent oxidation. At this point the proteins are ready to be immediately reconstituted with suitable solvent for further analysis.

2.8.6 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The following solutions were required for preparing separating gel mixture and stacking gel mixture:

Protogel Ultrapure (National diagnostics, Hessle Hull, England): 30% w/v acrylamide, 0.8% bisacrylamide stock solution (37.5:1).

Separating gel buffer: 1g, SDS and 45g, Tris buffer (2-amino-2-(hydroxymethyl)-propane-1,3-diol) dissolved in 200mL distilled water, pH was adjusted to 8.8 with HCl and volume made up to 250mL with distilled water.

Stacking gel buffer: 1g, SDS and 15.1g, Tris buffer dissolved in 200mL distilled water, pH was adjusted to pH 6.8 with HCl and volume was made up to 250mL with distilled water.

Ammonium persulfate solution: 1g, ammonium persulfate dissolved in 10mL distilled water.

2.8.6.1 Preparation of 10% separating gel mixture

10mL of Protogel, 12mL of distilled water and 7.5mL of separating gel buffer were mixed together and degassed using vacuum. 45µL of ammonium persulfate was added to the mixture, mixed gently and followed by 15 µL of TEMED (N,N,N',N', tetramethylethylene diamine) to start polymerisation. The mixture was mixed gently and

immediately poured into a previously prepared 0.75mm gel cast (Mini PROTEAN 3 system, Bio-Rad, Hercules, CA, USA). Distilled water was overlaid on the top of the gel to produce a flat top of the gel and to remove air bubbles. The gel was left to set at room temperature.

2.8.6.2 Preparation of stacking gel mixture

750 μ L of Protogel, 3mL of distilled water and 1.25mL of stacking gel buffer were mixed and degassed using vacuum. Then, 15 μ L of ammonium persulfate was added mixed gently and followed by 5 μ L TEMED. The mixture was mixed gently. After leaving the separating gel for at least one hour to polymerise, the overlaying water layer was decanted and the surface of the gel was rinsed with distilled water. Stacking gel mixture was poured over the separating gel and a well-forming comb (0.75mm, 10 wells, Bio-Rad) was inserted. The gel was left to set at room temperature for at least one hour.

Once the stacking gel had polymerised, the comb was removed and the wells were rinsed with distilled water. The gel was transferred to mini PROTEAN 3 system (Bio-Rad, Hercules, CA, USA) and the tank was filled with tank buffer (glycine (14.4g), Tris buffer (3.0g) and SDS (1.0g), distilled water added to 1L, pH about 8.3 without adjustment) so that the buffer covers the wells.

The samples and marker were loaded into the wells using special tips (Gel saver II Tips, Fisherbrand) and the tank was connected to electrophoresis power supply (Power 300, Fisher Scientific Labosi, France) to provide constant current (30mA). Once the bromophenol blue in the sample buffer/sample mixture migrated to reach 3 to 5mm from the bottom of the gel, the power was switched off. The gel was removed and immersed in fixing solution to be silver stained.

2.8.6.3 Preparation of sample buffer (double strength) (Smith, 1984; Boyer, 2000)

Sample buffer is used to denature protein sample and make it suitable for use with SDS-PAGE. It is used by mixing the sample with sample buffer in 1:1 ratio. Sample buffer is composed of reducing agent (β -mercaptoethanol), which breaks up disulfide bonds in the protein. SDS, anionic detergent that disrupts the secondary, tertiary, and quaternary structure of the protein to produce linear polypeptide chains. These chains are coated with negative charged SDS molecules. Bromophenol blue a dye that helps in tracking the migration of the sample during electrophoresis. Glycerol, since it has higher density than water, it makes the sample settles down the well during sample application rather than being mixed with tank buffer. Sample buffer was prepared according to Laemmli's method (1970) as follows:

Bromophenol blue (2mg), β -mercaptoethanol (2mL), glycerol (4.0g), SDS (0.92g), trizma base (4.0g), water added up to 10mL. pH adjusted to 6.8 by 5M HCl then volume completed to 20mL by water.

2.8.7 Protocol for preparing and running Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

2.8.7.1 Preparation of lysis / rehydration buffer (Anon, 2004)

The solution in which the sample will be dissolved before isoelectric focusing (IEF), should ensure complete solubilization and disaggregation of the proteins. Therefore, the solution should contain certain components in order to achieve well focused IEF separation. Sample solutions such as Lysis buffer and rehydration buffer usually contain urea and detergents. Urea is used as denaturant. It solubilizes and unfolds protein to expose it to the solution. A non-ionic detergent (such as 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS)) is also used to

ensure complete sample solubilization and to prevent hydrophobic interactions leading to aggregation. In order to reduce disulfide bonds and maintain the proteins in their reduced state, a reducing agent (dithiothreitol) is added. IPG buffer (GE health care, Sweden) can be added to improve protein solubility by decreasing charge-charge interactions and to ensure even charge distribution all through the pH gradient during first dimension analysis.

Since the composition of rehydration buffer is very similar to lysis buffer, one buffer was prepared, distributed in small Eppendorf tubes and stored at -20°C, until needed. Before use, the buffer was molten by leaving it at room temperature or by holding the tube by hand (because at temperatures above 37°C, the risk of protein carbamylation due to urea decomposition increases). After melting the buffer, some additives were added according to the buffer specific use (lysis or rehydration) as described below. The buffer was prepared by dissolving urea (19.2g) in distilled water up to 35mL, then CHAPS (1.6g) and 800µL IPG buffer (GE Healthcare, Sweden) were added and dissolved. The volume was completed to 40mL by distilled water.

Before using this buffer as lysis buffer, protease inhibitors (PMSF and TLCK) were added to achieve a final concentration of 1mM and 0.5mM respectively. Before using the buffer as rehydration buffer, bromophenol blue 1% w/v solution was added (20µL per 1mL lysis buffer) and dithiothreitol (DTT) was added to give a final concentration of 0.28% w/v.

2.8.7.2 Preparation of agarose sealing solution

In a 250mL Erlenmeyer flask, agarose (0.5g) and 200µL of bromophenol blue (0.002% w/v) were added to 100mL of tank buffer. The flask was placed on heating stirrer and the contents were heated until agarose was completely dissolved. The solution

was not allowed to boil. The contents were distributed in 2mL aliquots into screw cap tubes and stored at room temperature until needed (Anon, 2004).

Before use, the agarose tube was heated to melt the gel, then it was cooled to about 50°C before application on IPG strip on the top of the polyacrylamide gel.

2.8.7.3 Rehydrating the Immobiline pH gradient (IPG) strip (Anon, 2004)

Aliquot (125µL) of sample solution in rehydration buffer were pipetted into the slot of IPG re-swelling tray. The backing of IPG strip (Immobiline dry strip pH 3-10NL, 7cm, GE Healthcare) was peeled off and the strip was placed over the solution, with the gel side facing the solution, carefully so as not to trap air bubbles. The strip was overlaid with mineral oil (DryStrip Cover fluid, Amersham Biosciences) to minimise evaporation and urea crystallization. The tray was covered and the IPG strip was left to rehydrate at room temperature for 12 to 20 hours.

2.8.7.4 Running the first dimension (isoelectric focusing)

The rehydrated IPG strip was washed briefly with water and laid in a groove of the Immobiline DryStrip tray with the gel layer upward and the positive side toward the anode of the tank. The tray was previously prepared according to manufacturer's instructions and positioned on the Immobiline DryStrip Tank (Multiphor II, Amersham Biosciences). The tank was connected to thermostatic circulator (LKB Bromma, model 2219 Multitemp II, Sweden) to maintain the temperature at 20°C since heat will be generated from high voltage. Two damped isoelectric focusing electrode strips (IEF electrode strips, GE Healthcare) were placed just contacting each end of the IPG strip. Then, the suitable electrodes (anode and cathode) were positioned over the IEF strips. DryStrip Cover fluid (Amersham Biosciences) was poured in the tray to cover the strip.

The tank was connected to electrophoresis power supply (Model EPS-3501XL, Amersham Biosciences) to provide gradient voltage program at 1mA current divided into three phases. Phase one for 1min, voltage from zero to 200 volt, phase two for 90min, voltage from 200 to 3500 volt and the third phase for 55min at 3500 volt.

2.8.7.5 Preparation and running the second dimension

Protogel 10mL (30% w/v acrylamide, 0.8% bisacrylamide stock solution (37.5:1)), separating gel buffer 7.5mL (1g, SDS and 45g, Tris buffer (2-amino-2-(hydroxymethyl)-propane-1,3-diol) dissolved in 200mL distilled water, pH was adjusted to 8.8 with HCl and volume made up to 250mL with distilled water), and 12mL distilled water were mixed together and degassed using vacuum. Ammonium persulfate solution 45μL (1g, ammonium persulfate dissolved in 10mL distilled water) was added, mixed gently and followed by TEMED (N,N,N',N', tetramethylethylene diamine), 15μL to initiate polymerisation. The mixture was mixed gently and immediately poured into a previously prepared 1.0mm gel cast (Mini PROTEAN 3 system, Bio-Rad). Distilled water was overlaid on the top of the gel to produce a flat top of the gel and to remove air bubbles. The gel was left to set at room temperature for at least one hour. The water on the surface of the gel was decanted and the top of gel rinsed with water. The rehydrated IPG strip was inserted between the glass plates to rest on the top of the gel, after it had been washed with tank buffer (glycine (14.4g), Tris buffer (3.0g) and SDS (1.0g), distilled water added to 1L, pH about 8.3 without adjustment) for 15min with shaking. Warm liquid agarose sealing solution was pipetted over the IPG strip to form a sealing layer. The gel was transferred to mini PROTEAN 3 system (Bio-Rad) and the tank was filled with tank buffer so that the buffer covers the agarose layer. The tank was connected to an electrophoresis power supply (Power 300, Fisher Scientific Labosi, France) to

provide constant current (30 mA). Once the bromophenol blue front migrated to reach 3 to 5mm from the bottom of the gel, the power was switched off. The gel was removed and immersed in fixing solution to be silver stained.

2.8.7.6 Preparing and running 2D-PAGE markers

Markers for two dimensional electrophoresis (Sigma) (isoelectric point range (pI) 7.6-3.8; molecular weight range (M_r) 17000 to 89000 Da) (Table 2.1) were analysed by 2D-PAGE. According to supplier instructions, the marker was diluted 1/10 using rehydration buffer. 10 μ L of the diluted marker were mixed with 115 μ L of rehydration buffer. The mixture (125 μ L) was used to rehydrate Immobiline dry strip for 20hrs. IPG strips used covered the range of pH values from 3.5 to 10 (nonlinear range). After running the first dimension, the strip was washed with tank buffer and laid on top of 1mm previously prepared gel, sealed by agarose sealing solution and the second dimension was run. The gel was silver stained.

Table 2.1: composition of two dimensional electrophoresis markers (Sigma).		
Protein	Approximate pI	Approximate M_r (Da)
Amyloglucosidase from (<i>Aspergillus niger</i>)	3.8	89000; 70000
Ovalbumin	5.1	45000
Carbonic anhydrase from human erythrocytes	7.0	29000
Myoglobin from horse heart	7.6	17000

After staining the gels, retardation factor R_f was calculated for each protein spot in both dimensions (Table 2.2) according to the equation

$$R_f = \text{Distance travelled by the protein} / \text{Distance travelled by the dye front}$$

Then, two graphs were plotted. For the first dimension (pI dimension), Rf versus pI was plotted (Figure 2.7). For the second dimension (M_r dimension), Rf was plotted versus molecular weight (Figure 2.8). These calibration plots were used to estimate (pI) and (M_r) of all proteins analysed by 2D-PAGE through this study.

Table 2.2: Measurement data of two dimensional electrophoresis protein markers, indicating calculated retardation factor (Rf) of isoelectric point (pI) dimension and calculated Rf of molecular weight (M_r) dimension.			
pI	Rf of 1st dimension	M_r (kDa)	Rf of 2nd dimension
3.8	0.87	89,000; 70,000	0.18; 0.30
5.1	0.73	45,000	0.52
7.0	0.4	29,000	0.69
7.6	0.32	17,000	1

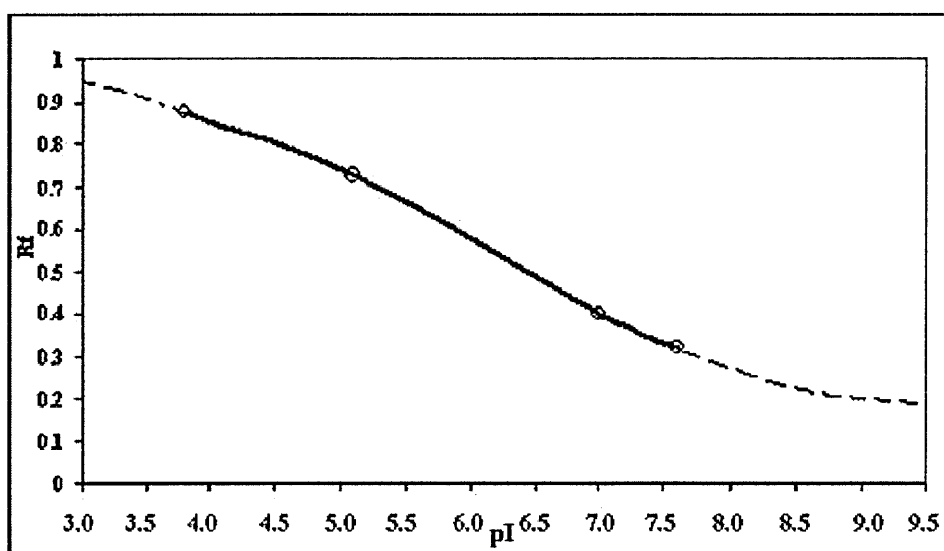


Figure 2.7: 2D-PAGE markers (Sigma). Rf versus isoelectric point (pI). Data points extrapolated were connected with discontinuous line. Data obtained from table 2.2.

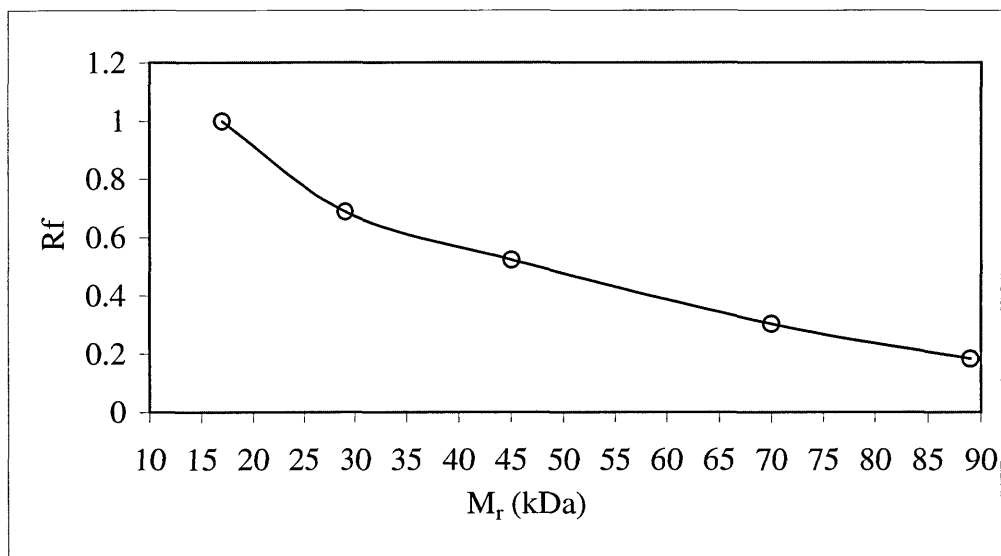


Figure 2.8: 2D-PAGE markers (Sigma). R_f versus molecular weight (M_r). Data obtained from table 2.2.

2.8.8 Preparation of solutions and procedure for silver staining

The following solutions were prepared for silver staining and were used according to the sequence below (Walker, 1984):

Fixing solution: (methanol, 100mL; glacial acetic acid, 24mL; 37% formaldehyde, 100 μ L; the volume was completed to 200mL with distilled water).

Wash A: 50% v/v ethanol.

Pre-treatment solution: (0.04g sodium thiosulfate pentahydrate in 200mL distilled water).

Impregnate solution: (0.4g silver nitrate (anhydrous), 37% formaldehyde, 150 μ L and distilled water up to 200mL).

Developing solution: (12g of sodium carbonate (anhydrous); 37% formaldehyde, 100 μ L, 4mL of pre-treatment solution and the volume was made up to 200mL with distilled water).

Stop solution: (100mL methanol, 24mL glacial acetic acid and distilled water up to 200mL). After immersing the gel in fixing solution for 1 to 16 hours, the solution was

decanted and wash A was added to the gel for 20min with shaking. This step was repeated another two times. Wash A was replaced by pre-treatment solution for one minute with shaking, then the solution was decanted and the gel was washed with distilled water three times each time for 20 seconds with shaking. Water was decanted and impregnate solution was added to the gel for 20 min and then washed twice with distilled water each time for 20 seconds. Then the gel was transferred to developing solution until the bands or spots were clearly visible. Immediately the gel was washed with distilled water for 20 seconds and was transferred to stop solution for ten minutes to stop staining reaction. The gel can be stored in distilled water at 4°C.

2.8.9 Checking the possibility of interference from brain heart infusion in SDS-PAGE.

Since BHI is a complex medium that has high concentration of proteins, the possibility of having interference with SDS-PAGE analysis was checked. BHI medium was prepared as described previously from two different bottles. An aliquot (100µL) of the prepared medium was mixed with equal volume of sample buffer and heated for 5 minutes at 90°C. Samples (25µL aliquots) were applied on previously prepared gels. After running the gels under constant current (30 mA), they were silver stained. Figure 2.9 shows SDS-PAGE analysis of BHI. No protein bands appeared in the region of 10 to 150kDa.

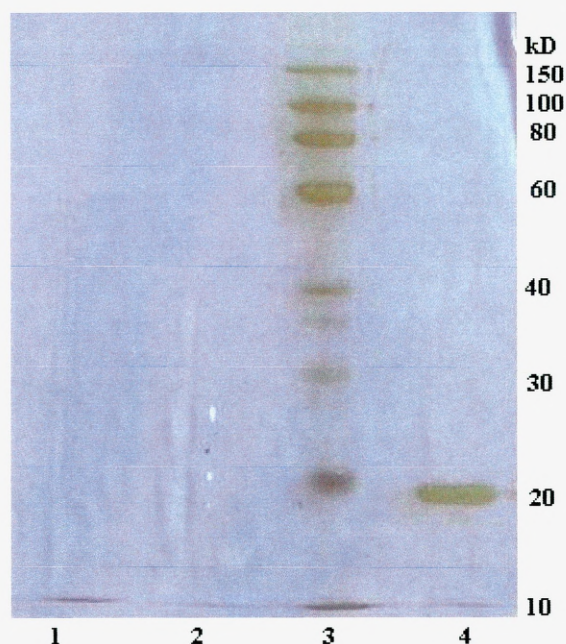


Figure 2.9: SDS-PAGE analysis of two BHI media. Lanes (1) and (2), BHI media from two different bottles; lane (3), SDS-PAGE marker and lane (4), TSST-1 standard (Sigma).

2.9 Virulence factors of *S. aureus* 11962

2.9.1 Determining coagulase titre

Rabbit plasma (lyophilised and citrated) (Sigma) was reconstituted in sterile distilled water according to manufacturer instructions and a solution of 20% v/v was prepared.

BHI (80μL) was added to each well of 96-well round bottom microtiter plates, leaving the first row of wells empty. Eluate supernatants (80μL) were placed in triplicate in the first row and the second row of 96-well round bottom microtiter plates and the contents were mixed. Double serial dilutions of eluate supernatants were done in BHI in the range of 2 to 1.3×10^5 starting from the second row to the next row by transferring 80μL and mixing. Equal volumes of rabbit plasma were added and mixed with eluate supernatants in the wells. Negative control was prepared by mixing brain heart infusion medium with the

plasma. The plates were covered and incubated at 37°C. After 4 hours, the plates were inspected visually against a black background for coagulation.

2.9.2 Biofilm assay in microtiter plate

2.9.2.1 Preparation of Gram stain Hucker's modification

Crystal violet (2g) was dissolved in 96% ethanol to make 20.0mL. The solution was added to 80.0mL of ammonium oxalate solution (1% w/v).

2.9.2.2 Biofilm assay

The method used by Stepanovic *et al.*, (2000) was applied. Stock solutions of antibiotics were prepared in BHI. The concentration of these stocks were adjusted so that when 90 µL aliquots were mixed with 20 µL bacterial suspension the final concentration of antibiotics would be equivalent to 90% of MIC.

Aliquots (90µL) of antibiotics solutions in BHI were placed in triplicate in sterile 96-well round bottom microtiter plates. Double serial dilutions were performed to cover the range from 90% MIC to 0.35% MIC of the final concentration. Aliquots (90µL) of BHI were placed in other wells as controls. Aliquots (20µL) of bacterial culture in mid exponential phase (adjusted to 0.5 McFarland absorbance) were added to all the wells including the control wells. The plates were covered and incubated for 24hr at 37°C. The contents of the wells were aspirated and the wells were washed with 150µL sterile normal saline (0.9% w/v) with vigorous shaking. The contents were aspirated and each well was washed three times more with 150µL sterile normal saline. In order to fix the remaining attached bacteria, 90µL of methanol (99%) was added to the wells and left for 15 minutes, then removed by decanting. After the wells dried, Hucker's crystal violet (71 µL) was added to each well and left for 5 minutes then discarded. Excess stain was

rinsed off by washing the plate with plenty of running water until water is colourless and then the plate was left to air dry. In order to solubilize the dye bound to biofilms, 160 μL of glacial acetic acid 33% v/v was added to the wells and the plate was placed in ELISA reader (Rosys anthos ht III, Anthos Labtec Instruments, Salzburg, Austria) to measure the absorbance at 562nm after shaking the plate at medium speed for 30 seconds.

2.9.3 Protease test

2.9.3.1 Zymography

Zymography is an electrophoretic technique to detect for proteolytic activity in polyacrylamide gels. Sample buffer (5x strength) consisting of 0.4M Tris base, SDS (5% w/v), glycerol (20% w/w) and bromophenol blue (0.03% w/v), final pH 6.8 adjusted by 5M HCl, was prepared to dilute the samples (Kleiner and Stetler-Stevenson, 1994). Separating gel containing 0.5mg mL^{-1} casein was prepared by dissolving 14.8mg casein in 12mL of distilled water and 7.5mL of separating gel buffer. Protogel (10mL) was added and the solution was mixed together and degassed using vacuum. Ammonium persulfate (45 μL) was added to the mixture, mixed gently and followed by 15 μL of TEMED (N,N,N',N', tetramethylethylene diamine) to start polymerisation. The mixture was mixed gently and immediately poured into a previously prepared 0.75mm gel cast (Mini PROTEAN 3 system, Bio-Rad, Hercules, CA, USA). Distilled water was overlaid on the top of the gel to produce a flat top of the gel and to remove air bubbles. The gel was left to set at room temperature. Stacking gel was prepared as described in Section 2.8.6.2 and poured on top of the separating gel. Tank buffer was prepared as described in Section 2.8.6.2. When stacking gel has polymerised, samples were applied to the gel and constant current (35 mA) applied. Once the dye front migrated to the bottom of the gel, the gel was removed and incubated for 1 hour at room temperature with shaking in

detergent solution (composed of 2.5% v/v Triton X-100; Kleiner and Stetler-Stevenson, 1994; Leber and Balkwill, 1997). The Triton X-100 solution was decanted and the gel was washed twice in enzyme buffer (50mM Tris, pH7.6, 200mM NaCl, 5mM CaCl₂, 0.25% v/v Triton X-100; King *et al.*, 1996), then incubated in the same buffer for 18hr at 37°C. After brief rinsing in distilled water, the gel was stained in Coomassie brilliant blue R-250 stain (0.2g coomassie brilliant blue, 125mL methanol, 25mL glacial acetic acid and 100mL distilled water) for 4 hours at room temperature with gentle shaking. Destaining was performed by several changes of destain solution (450mL methanol, 100mL glacial acetic acid and 450mL distilled water) on the gel until colourless areas of substrate degradation appeared.

2.9.3.2 Azocasein assay

The method used by Miedzobrodzki *et al.*, (2002) was used with slight modification. An aliquot (50µL) of the sample was added to 750µL incubation mixture (0.1M tris-HCl buffer, pH 7.8, 450µL and 300µL of 1.5% w/v azocasein in water) in an Eppendorf tube. The contents were vortexed and incubated for three hours at 37°C. The enzymatic reaction was stopped by adding 800µL of 6% w/v trichloroacetic acid and mixing. The precipitated unreacted protein was removed by centrifugation for 20 minutes at 13000 rpm (Genfuge 24D, Progen, Mexborough, UK). The absorbance of the released acid-soluble azo-peptide was measured at 365nm in quartz cuvette.

2.9.4 Assaying TSST-1 in eluate supernatant

2.9.4.1 Determining the titre of TSST-1 in eluate supernatant

This test was performed using Toxin detection kit (TST-RPLA Staphylococcal Toxic Shock Toxin, Oxoid, UK) according to manufacturer's instructions. RPLA diluent

(25µL aliquots) were added into the wells of 96-well V-shaped bottom microtiter plate leaving the first row wells empty. Eluate supernatants of each treatment were added in 25µL aliquots, in triplicate, into the first and second row and mixed. Double dilution was performed by transferring 25µL from the second row wells to the next one with mixing. This was repeated until serial dilutions in the range (5×10^{-1} to 1.5×10^{-5}) were achieved. To these wells, 25 µL of latex suspension sensitised with specific rabbit IgG antibody against staphylococcal TSST-1 was added. Two negative controls were prepared; the first one by mixing equal volumes (25µL) of latex suspension sensitised by non-immune rabbit serum with all samples' dilutions, and the second one by mixing equal volumes (25 µL) of latex suspension sensitised with specific rabbit IgG antibody against staphylococcal TSST-1 with RPLA diluent. On the other hand, purified TSST-1 provided by the kit served as positive control.

The plates were mixed, covered with lids and incubated for 24hr at room temperature on a vibration free surface. After incubation, the plates were visualised against black background for agglutination and results were interpreted according to manufacturer's criteria. The highest dilution of supernatant showing agglutination was considered the titre.

2.10 Protocols for toxin purification.

In this project, Toxic Shock Syndrome Toxin 1 (TSST-1) purification from eluate supernatant was done using column chromatography techniques; ion exchange and gel chromatography in addition to other steps for desalting and concentrating the protein. The following buffer solutions were required for purification steps and prepared in ultra pure water ($18 \Omega \text{ cm}^{-1}$).

5mM sodium phosphate buffer pH 5.6: 0.6545g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.069g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and water added up to 1L.

0.5M sodium phosphate buffer with 0.5M NaCl pH 6.2 : 38.99g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 58.28g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 29.22 g NaCl; water added to less than 1L, pH adjusted with 1M NaOH (if necessary) and volume completed to 1L with water.

5mM sodium phosphate buffer pH 6.0: 0.605g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 0.165 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and water added up to 1L.

0.03M sodium phosphate buffer pH 6.0: 3.63g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 0.988g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; water added to less than 1L, pH adjusted with 1M NaOH (if necessary) and volume completed to 1L with water.

0.045M sodium phosphate buffer pH 6.4: 4.564 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 3.20g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and water added to less than 1L, pH adjusted with 1M NaOH (if necessary) and volume completed to 1L with water.

0.05M sodium phosphate buffer pH 6.8 containing 1M NaCl: 3.52g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 6.566g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 58.44g NaCl; 900mL water added, pH adjusted to 6.8 by 5M NaOH and volume completed to 1L with water.

5mM sodium phosphate buffer pH 6.8: 0.371g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 0.62g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and water added up to 1L.

2.10.1 Preparation of Amberlite CG-50 ion exchanger

Amberlite CG50 (Sigma) is a weakly acidic cationic exchanger. Its matrix is composed of 4% cross linked methacrylate with carboxylic acid functional group. A quantity equal to 210mL wet resin was washed with 0.01M NaOH followed by washing with 0.01M HCl. The resin was then washed several times and equilibrated with 5mM sodium phosphate buffer pH 5.6.

2.10.2 Preparation of ion exchange column CM-Sepharose CL 6B

CM-Sepharose CL 6B (Sigma) is a cationic exchanger. It is composed of carboxy methyl functional groups attached to cross linked agarose gel Sepharose CL-6B. CM-Sepharose CL 6B is supplied pre-swollen in 20% ethanol. A sufficient quantity was poured in a beaker and equilibrated to room temperature. The solvent was decanted and replaced with 5mM sodium phosphate buffer pH 5.6 (previously filtered through 0.45 μ m membrane filter) to form 75% settled medium to 25% buffer. The slurry was degassed under vacuum. The ion exchanger mixture was mixed by swirling the beaker and poured slowly in XK column 26/40 (GE Healthcare) already mounted vertically and contained few millilitres of the buffer. The ion exchanger was left to settle down, excess buffer was removed by aspiration and additional ion exchanger-buffer mixture was poured slowly. The process was repeated until a column bed height of 38 to 40cm settled ion exchanger is reached. Then, the 5mM sodium phosphate buffer pH 5.6 was pumped at a flow rate of 120mL hr⁻¹ from a reservoir to the top of the column using a pump (Pharmacia, model P-500, Sweden). The column was allowed to condition with buffer until the conductivity and pH of the buffer from the column outlet equals that of the fresh buffer (after approximately two days).

2.10.3 Preparation of Gel filtration column Sephacryl S200 HR

Sephacryl S200 HR (Sigma) is a hydrophilic matrix composed of cross linked allyl dextran with N,N'-methylene bisacrylamide. It is supplied pre-swollen in 20% ethanol. A sufficient quantity equivalent to 115% of the required bed volume was poured in a beaker and equilibrated to room temperature. The solvent was decanted and the gel was washed twice with degassed and filtered (0.45 μ m) water. Then the gel was suspended in water to form 75% settled medium to 25% water. The slurry was degassed

under vacuum. A packing reservoir was attached to a column XK 26/100 (GE Healthcare) and mounted vertically. The whole amount of gel mixture was mixed by swirling the beaker and poured slowly in the column, which already contained few millilitres of water. Water was pumped from solvent reservoir to the top of the packing reservoir at flow rate 180mL hr^{-1} for two hours, and then the flow rate was increased to 320mL hr^{-1} for one hour. Excess water was removed from the top of the gel bed and the column adapter was connected. The column was then washed by delivering ten column volumes of 0.05M sodium phosphate buffer pH 6.8 containing 1M NaCl at FR 60mL hr^{-1} .

2.11 Statistical analysis

All tests were performed in triplicate and the average result was recorded. Standard deviation of the data points from the mean was calculated.

Where stated, one way analysis of variance (ANOVA) was performed for multiple comparisons followed by Tukey multiple intergroup comparison. P values <0.05 were considered significant. Normality of results was tested using Anderson-Darling test ($p >0.05$). Equal variance was tested using Bartlett's test ($p >0.05$). All calculations were carried out using Minitab 15 software.

Chapter 3

Development of suitable biofilm growth regimes and initial antibiotic sensitivity testing for *Staphylococcus aureus* and *Staphylococcus epidermidis*

3.1 Introduction

Staphylococci are natural colonizers in animals and humans. The number of infections caused by them is minor when compared to their widespread nature. *S. aureus* preferentially colonizes the anterior nares and is also isolated from healthy skin and throat (Kluytmans *et al.*, 1997; Casey *et al.*, 2007). *S. epidermidis* mainly colonizes the skin and is considered one of the predominant human skin flora (Casey *et al.*, 2007). Staphylococci are chemoheterotrophs that require organic carbon as their source of energy. Nutritional requirements of staphylococci are generally complex and depend on the strain (Keller *et al.*, 1978; Onoue and Mori, 1997). Several investigators had shown that the components and their concentrations in a culture medium have great impact on the growth and virulence of *Staphylococcus aureus* (enterotoxin and capsule polysaccharide production) (Bhaduri, 1983; Stringfellow *et al.*, 1991; Onoue and Mori, 1997).

In general, synthetic chemically defined media are preferred to complex media because chemically defined media guarantee reproducibility in composition, support growth at suitable rates and avoid unnecessary excess nutrients, which in turn reduces or avoids unwanted interactions that may occur with other materials added (Foucaud *et al.*, 1997).

The objective of this section is to find a suitable medium that supports the growth of *S. aureus* and *S. epidermidis* in planktonic and biofilm mode, for use in subsequent experiments. Initially, the suitability of a chemically defined medium CDM (Dinning,

1995; Al-Hmoud, 2002; Masadeh, 2005; described in Chapter 2) to support the growth of *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 was investigated. This medium includes an organic source of carbon (glycerol) and a source of nitrogen (ammonium chloride and ferric ammonium citrate). It also has chloride, calcium, potassium, magnesium and iron sources.

3.2 Assessing Bacterial growth

Several methods have been used to measure bacterial growth (Fuchs and Kroger, 1999). The most widely used ones are the viable count and turbidimetric methods. Viable count is used to estimate the viable population in a bacterial culture. In this method, a bacterial suspension is serially diluted and plated onto solid medium in order to determine the number of colonies after each incubation period. Each colony is assumed to arise from individual bacterial cell. Therefore, by counting the number of colonies and knowing the dilution factor, colony forming units (CFU) can be determined (Salvesen and Vadstein, 2000).

Bacterial cell size lies within the order of wavelength of visible light. Therefore, it has been shown that measuring the amount of visible light scattered or absorbed by bacterial cells can be used to estimate bacterial concentration (Fuchs and Kroger, 1999). At low cell densities, the optical density (absorbency) is proportional to bacterial concentration. Since secondary light scattering increases as particles concentration increases, it is necessary to dilute bacterial suspensions with optical densities above 0.5 (Lawrence and Maier, 1977).

3.3 Experimental approach

3.3.1 Preparation of inoculum culture

A sterile 100mL Erlenmeyer flask containing 25mL of sterile medium was inoculated with pure culture of the bacteria to be studied. The flask was incubated in a shaking incubator at 150 osc min⁻¹ (Infors Ecotron AG CH-4103, Belgium) at 37°C overnight.

3.3.2 The test culture

Sterile 100mL Erlenmeyer flasks containing 25mL of fresh medium were inoculated with 250μL of inoculum culture. The flasks were incubated in shaking incubator 150 osc min⁻¹ at 37°C. Two test cultures were prepared; one for viable count test the other for turbidimetric assay.

3.3.3 Viable count

At hourly intervals, an aliquot (100μL) of test culture was removed and serially diluted in normal saline (0.9% w/v) in the range of 10⁻² to 10⁻⁸. Aliquots (0.1mL) of the dilutions were spread plated onto tryptone soya agar plates (pre-dried at 60°C for 5minutes). The plates were prepared in triplicate for each dilution. After 48hr incubation at 37°C, colonies were counted in each plate and viable count was calculated by the following equation:

$$\text{CFU mL}^{-1} = N(1/\text{DF}) \quad \text{equation 3.1}$$

Where N is the average number of colonies on the three plates (it should be between 30-300 to be statistically valid) and DF is the dilution factor (taking into account the dilutions and the volume used on the spread plate).

3.3.4 Turbidimetric growth assay

An aliquot (1mL) of test culture was removed aseptically at hourly intervals. The optical density (OD) was measured at 470nm using spectrophotometer (Genesys 10UV Thermo Spectronic, USA). Fresh medium was used as a blank. After measurement, the sample was aseptically returned to the culture flask so as not to alter the culture volume significantly. If OD values were above 0.5, a minimal volume (0.1mL) of culture was removed and diluted in fresh medium to bring OD below 0.5 (Lawrence and Maier, 1977). The diluted cultures were discarded after measuring their OD. The optical density of the diluted samples was calculated by multiplying OD by the reciprocal of the dilution factor.

3.3.5 Modification of chemically modified medium (CDM)

CDM was prepared and sterilized as mentioned in Chapter 2. Several modifications were performed on this medium. Whenever glucose was added, it was sterilized separately by autoclaving at 121°C, 15psi for 15 minutes. All amino acids used were of HCl salt except Glycine. Stock solutions of aminoacids were prepared by dissolving specific amount in 10mL water (Table 3.1) and then sterilized by autoclaving. For media preparation, 0.5mL of amino acid stock solution was aseptically added to 24.5mL of sterile CDM.

Table 3.1 : Weight of amino acids dissolved in 10mL water to prepare different formulations of CDM media.	
Amino acids	Weight (mg)
L-Valine (Val)	40
L-Arginine (Arg)	250
L-Cystine (Cys)	15
L-Aspartic acid (Asp)	45
Glycine (Gly)	25
L-Leucine (Leu)	45
L-Proline (pro)	40
L-Glutamic acid (Glu)	50
L-Tryptophan (Try)	15
L-Methionine (Met)	15
L-Isoleucine (Ile)	15
L-Histidine (His)	15

3.3.6 Results and discussion

Chemically defined medium (CDM) (Chapter 2) was used as a starting medium to study the growth of *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228. Figures 3.1 (A) and (B), present growth curves of *S. aureus* and *S. epidermidis* in CDM assayed by viable count and turbidimetric methods, respectively. In this medium, the lag phase of both bacterial cultures (Figure 3.1C) was around two hours. The exponential phase of *S. aureus*, which lasted for 3.5 hours, was much shorter than that of *S. epidermidis* which lasted for 15 hours. As a result of the shorter exponential phase, *S. aureus* entered the stationary phase much earlier than *S. epidermidis* (5hrs and 17hrs after inoculation, respectively). Bacterial growth yield, which reflects dry cell mass per unit volume, is defined as the final optical density (OD) minus the initial OD measured immediately after inoculation (Stringfellow *et al.*, 1991). Bacterial growth yield of *S. epidermidis* (OD_{470nm}

1.6) was much higher than that of *S. aureus* (OD_{470nm} 0.4). The generation time or doubling time for *S. epidermidis* (200 minutes) was about 20% greater than that of *S. aureus* (165 minutes). The same growth pattern of *S. aureus* ATCC 6538 was noticed in other strains of bacteria; *S. aureus* ATCC 27659 and NCTC 11962.

In order to improve the growth of *S. aureus*, many modifications to the CDM were investigated and for each modification, growth yield and / or growth curve was constructed and compared with the original one. Only one modification was done at a time maintaining the other parameters constant.

The initial steps performed were to change the composition of the medium. Doubling or tripling nitrogen content by increasing the amount of ammonium chloride in the medium did not improve growth yield. The same was noticed with magnesium and with carbon source (glycerol). Replacing the glycerol with glucose had no noticeable effect on the growth. Increasing the content of iron or changing its source by replacing ferric ammonium citrate with ferric chloride had no effect. Even when all sources of carbon, nitrogen, magnesium and iron were doubled or tripled growth yield remained the same. These results indicate that the components of this medium cannot support the growth of *S. aureus* and other nutritional sources should be tested.

Specific amino acids were shown to enhance the growth of *S. aureus* in different chemically defined media depending on the strain and media composition (Mah *et al.*, 1967; Keller *et al.*, 1978; Bhaduri, 1983). L-valine, L-arginine and L-cystine were found to be necessary for the growth of several *S. aureus* strains (Onoue and Mori, 1997). In order to determine aminoacid requirements, a series of CDM starting with these three aminoacids were prepared (Table 3.2). Each medium differed from the previous by addition of new aminoacid. Aminoacids investigated in this study were selected from previously published work (Mah *et al.*, 1967; Keller *et al.*, 1978; Bhaduri, 1983;

Stringfellow *et al.*, 1991) and the concentrations prepared were the same concentrations used by Onoue and Mori (1997), except that aminoacid concentrations were adjusted so that no final concentration was below 30µg mL⁻¹ (Keller *et al.*, 1978; Bhaduri, 1983) (Table 3.1). For reasons of simplicity, bacterial growth yield at OD 470nm was used to compare the results of different media formulations (Table 3.2).

In order to simplify the formulation of the medium, the CDM with lowest number of components giving highest OD was chosen for further work. Thus, the CDM with 7 aminoacid added (formula 5) was selected (referred to as CDM-7 throughout). Figure 3.2 shows comparison between growth curves of *S. aureus* ATCC 6538 in CDM and in CDM-7. Addition of amino acids increased the duration of exponential phase from 3.5 hours to 5 hours and the generation time was reduced from 165 minutes to 63 minutes. This was reflected in bacterial growth yield, which was improved from less than OD 0.5 to about OD 2.0. Other strains of *S. aureus* (ATCC 27659 and NCTC 11962) were tested using this medium and gave similar results.

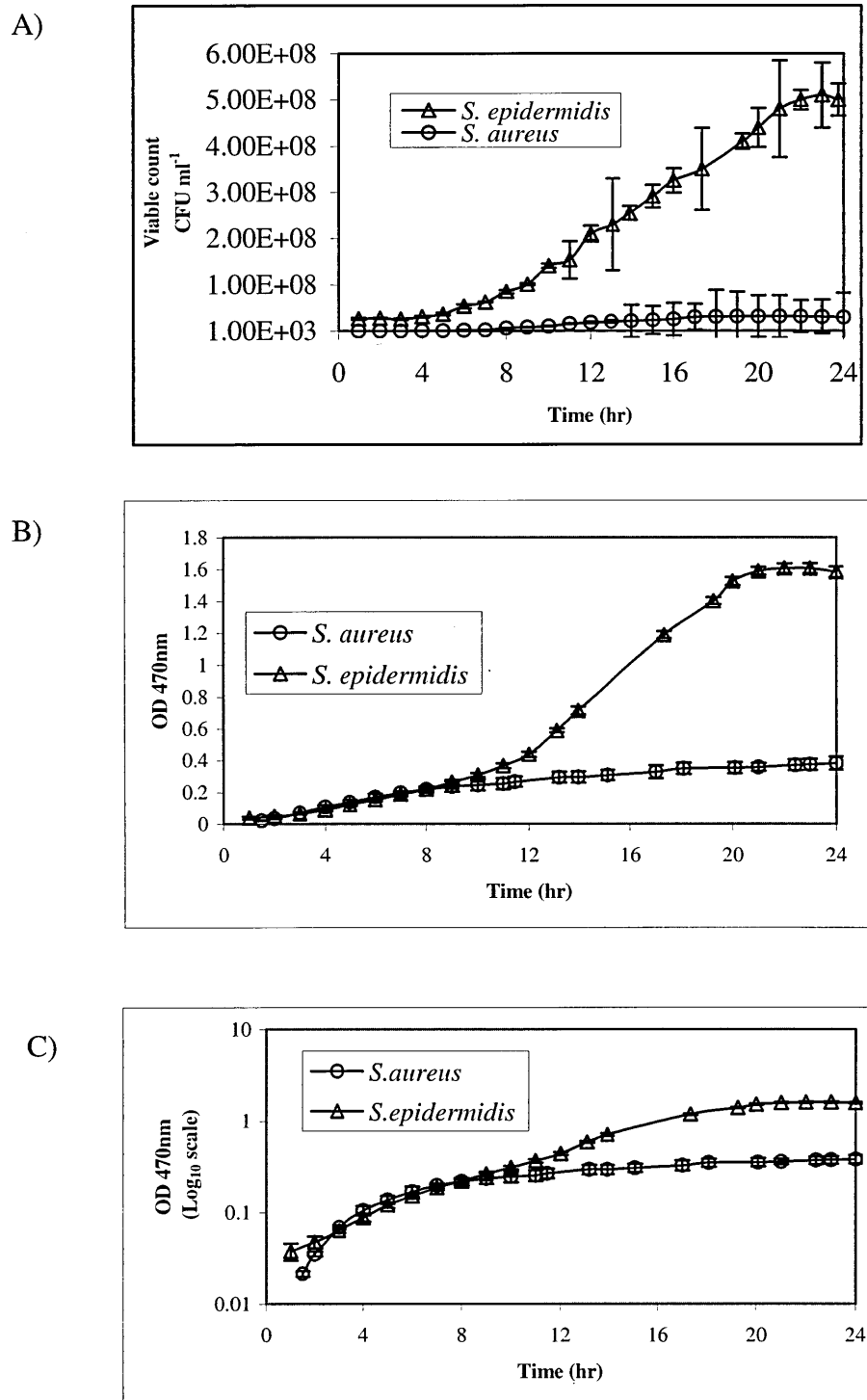


Figure 3.1: *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 growth curves in CDM. (A) mean viable count vs time. Non-logarithmic Y-axis gives non-linear details at values below 1.00E+08. (B) OD at 470nm vs time and (C) OD at 470nm vs time in log scale. Error bars indicate \pm standard deviation of individual points, where n=3.

Table 3.2: Different formulations of aminoacids were prepared; each one differs from the previous by addition of one new aminoacid to the basic CDM. Growth yield at 470nm was measured and used to compare between the formulations. (For concentrations and abbreviation of aminoacids see table 3.1).

Formula number	Medium	Growth yield OD_{470nm}
1	Val, Arg, Cys	0.601
2	Val, Arg, Cys, Asp	0.613
3	Val, Arg, Cys, Asp, Gly	0.615
4	Val, Arg, Cys, Asp, Gly, Leu	0.588
5	Val, Arg, Cys, Asp, Gly, Leu, Pro	1.965
6	Val, Arg, Cys, Asp, Gly, Leu, Pro, Glu	1.896
7	Val, Arg, Cys, Asp, Gly, Leu, Pro, Glu, Try	1.914
8	Val, Arg, Cys, Asp, Gly, Leu, Pro, Glu, Try, Met	1.968
9	Val, Arg, Cys, Asp, Gly, Leu, Pro, Glu, Try, Met, Ile	1.888
10	Val, Arg, Cys, Asp, Gly, Leu, Pro, Glu, Try, Met, Ile, Hist	1.921

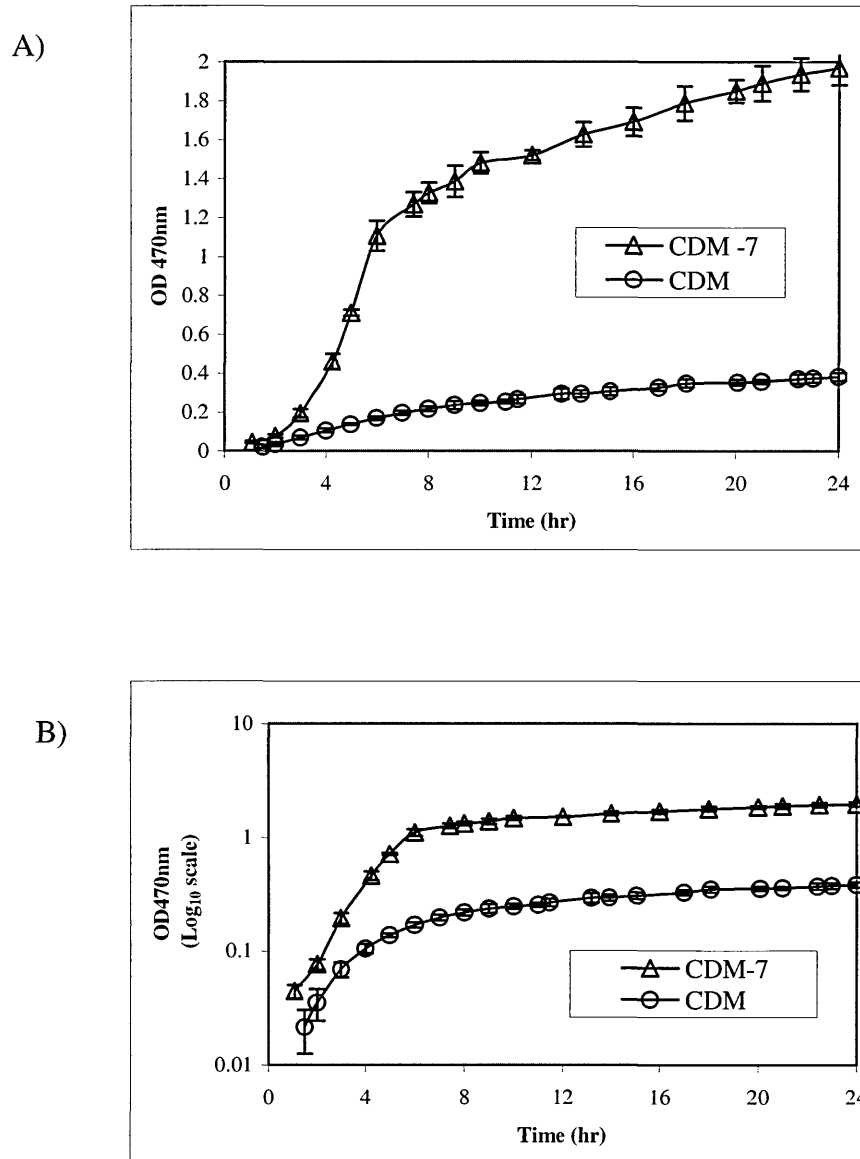


Figure 3.2: *Staphylococcus aureus* ATCC 6538 growth curve in CDM and CDM-7 medium. Optical density at 470nm vs time. (A) linear scale, (B) log scale. Error bars indicate mean \pm standard deviation of individual data points, n=3.

This work and the work of others (Mah *et al.*, 1967; Keller *et al.*, 1978; Bhaduri, 1983; Onoue and Mori, 1997) indicates that in order to support the growth of *S. aureus* in chemically defined medium, other sources of nutrients are required in addition to the compound sources of carbon, nitrogen, iron, magnesium and other ion sources normally supplied. It has been found that addition of seven aminoacids (L-valine, L- argenine,

L-cystine, L-aspartic acid, glycine, L-leucine, L-proline) to the normal CDM formula was necessary to support the growth of *S. aureus* ATCC 6538, ATCC 27659 and NCTC 11962. On the other hand, CDM alone has been found to be sufficient to support *S. epidermidis* growth. Addition of the seven aminoacids has no effect on growth dynamics of *S. epidermidis* (data not shown). After formulating suitable medium for each bacterium, these media will be used to grow the bacteria in biofilm systems to be subjected to subsequent studies.

3.4 Molecular basis of biofilm development

Biofilm formation involves transformation of free flowing planktonic cells into a sessile population attached to a surface. This surface (substratum) is normally coated with a conditioning film resulting from the interaction of a surface with the medium (Dunne, 2002). Conditioning films are organic in nature and form within minutes after exposure to an overlying solution containing biological and biochemical components (Donlan, 2002). Their composition is variable depending on the surrounding environment. Biofilm formation passes through several stages that involve physico-chemical and biological interactions. These stages are discussed in the following sections.

1) Primary adhesion

The initial step in biofilm formation is the adherence of microbial cells to a surface. Microorganisms freely flowing in the bulk fluid could be brought into close proximity to a body or surface either randomly (for example by Brownian motion, convection by a stream or turbulence of fluid flowing over surface), or in a directed fashion by the chemotactic ability of the microorganisms and subsequent mobility (Donlan, 2002). When a microorganism reaches a distance of about 50nm from a

surface, electromagnetic forces initiate adhesion via long and short range forces (Gorman and Jones, 2006). Microorganisms can be regarded as living colloidal particles, hence, Derjaguin-Landau-Verwey-Overbeek (DLVO) hypothesis, which describes electromagnetic forces that attract and repulse colloidal particles, may be applied to them (Gorman and Jones, 2006).

According to this hypothesis, the net interaction, V_T , between a cell and a surface is a balance between attractive forces, V_A (Figure 3.3), resulting from London-type van der Waals attractions and repulsive interaction, V_R (Figure 3.3), resulting from the electrostatic interaction between charged bacterial cells and the surface (substratum). In general, bacterial cells and substratum are negatively charged at physiological pH (Hermansson, 1999). According to DLVO theory, the composite potential energy or the net interaction, V_T , of these forces will have a potential attraction near the origin, called the primary minimum (point of irreversible adhesion), and high potential barrier or repulsion (the positive maximum) at a moderate distance (Figure 3.3). In some cases, a shallow secondary attraction or minimum (reversible adhesion) is observed at longer distances of separation (Martin and Bustamante, 1993). Thus, when a negatively charged bacterium approaches a negatively charged surface, the composite potential energy is a function of separation and the ionic strength of the medium (Dickinson and Bisno, 1989).

For a microorganism to adhere to a surface, it has to move from a situation where the cell is reversibly attached in a secondary minimum, to reach the primary minimum (point of irreversible adhesion) and subsequently contact the substratum. This step requires that repulsive forces (positive maximum) should be overcome (Hermansson, 1999). The interfacial free energy of adhesion, ionic strength of the medium and presence of specific bacterial mechanisms for attachment are the key players in order

for adherence to occur (Dickinson and Bisno, 1989; Gorman and Jones, 2006). For adhesion between particles and surfaces to occur, the interfacial free energy of adhesion should be thermodynamically favourable (Strevett and Chen, 2003). In other words, (bacterium-surface) interaction should be thermodynamically more favourable than (bacterium-water) and (surface-water) interactions. Hydrophobic groups on the bacterial cell surface limit bacterium-water interactions thus, favouring bacterium-surface interactions. This effect may help explain the role of cell surface hydrophobicity in bacterial adhesion (Dickinson and Bisno, 1989). However, bacterial cell hydrophilicity or hydrophobicity depends largely on bacterial growth stage and the growth medium which may influence the process of attachment (Grasso *et al.*, 1996; Chen and Strevett, 2002). For example, the hydrophilicity and hydrophobicity of *Escherichia coli* has been shown to be altered depending on carbon and nitrogen source and on the abundance of these elements (Chen and Strevett, 2002). In stationary phase, *Pseudomonas aeruginosa* was found to have more hydrophobic surfaces and to significantly partition more to dolomite surfaces than in the exponential or decay phase (Grasso *et al.*, 1996).

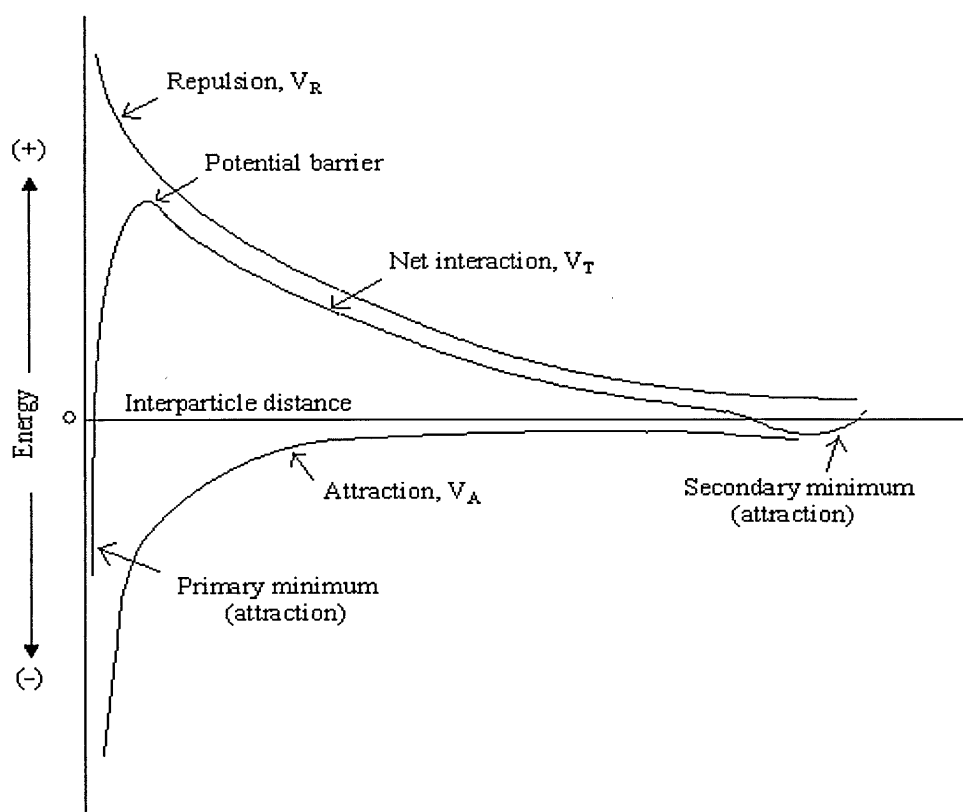


Figure 3.3: DLVO theory as applied to the adherence of microorganism to a substratum. V_R repulsive forces, V_A attractive forces and V_T net interaction (adapted from Martin and Bustamante, 1993).

On the other hand, the substratum hydrophobicity reduces surface-water interactions, thus, favouring bacterium-surface interactions. In a study, *P. aeruginosa* and *S. epidermidis* adhered more to hydrophobic type contact lenses than to hydrophilic types (Henriques *et al.*, 2005). The same observation was reported by Santos *et al.* (2007) where hydrophobic lenses were the highest in microbial colonization compared to other hydrophilic types.

Increasing ionic strength of the medium will reduce the positive maximum, allowing a greater proportion of microorganisms to reach primary minimum and adhere to the surface (Gorman and Jones, 2006). Adhesion of several strains of *P. aeruginosa*, *Burkholderia cepacia*, *E. coli* and *Bacillus subtilis* to inorganic surfaces was significantly increased when the ionic strength of the medium was increased from

1mM to 100mM (Li and Logan, 2004). Adherence of many strains of *S. epidermidis* to plastic has been shown to be enhanced by the presence of calcium and magnesium ions (Dunne and Burd, 1992). However, addition of ethylenediaminetetraacetic acid (EDTA) results in a dramatic decrease in their adherence (Dunne and Burd, 1992).

The other tactic used by microorganisms to overcome the potential barrier (positive maximum) is not accounted for by DLVO theory. For this step, microorganisms use their cell surface structures or molecules to bridge the gap between secondary minimum (distance range 20-100nm) and primary minimum (1nm) by attaching to specific substratum structures (Costerton *et al.*, 1999; Hermansson, 1999; Donlan, 2002). *S. aureus* may use its adhesin-like compounds (fibronectin binding protein, clumping factor, polysaccharide intercellular adhesin, etc.; Dickinson and Bisno, 1989; Que *et al.*, 2001) to attach to surfaces. Other bacteria use their fimbriae, pili or flagella for this purpose (O'Toole and Kolter, 1998a; Watnick and Kolter, 2000). *P. aeruginosa* mutants defective in flagellar-mediated motility, were found to exhibit a poor ability to attach to PVC surface when compared with the wild type. The wild type was able to form microcolonies that dispersed on the surface forming a monolayer (O'Toole and Kolter, 1998a). On the other hand, fimbriae have been found to increase cell surface hydrophobicity and therefore, to enhance bacterial adhesion to abiotic surfaces (Zita and Hermansson, 1997; Hermansson, 1999).

2) Microbial attachment to the surface and microcolony formation

The forces involved in the initial adhesion are reversible ones, and so far the microorganism is loosely bound to the surface (Costerton *et al.*, 1999). Subsequently, cells may become firmly attached to the surface through the action of surface structures. This irreversible attachment of the microorganism to the surface is

mediated by the adhesins of the microorganism and complementary receptors on the biomaterial surface (Gorman and Jones, 2006). After attaching to the surface, microorganisms start to multiply, adhere to each other and form microcolonies. Microcolonies are relatively small groups of bacteria and are considered the basic unit of biofilm growth (Costerton *et al.*, 1995; Costerton *et al.*, 1999; Watnick and Kolter, 2000).

3) Biofilm maturation

In this stage, the complexity, heterogeneity and density of biofilms increase. Microorganisms bound to the surface replicate and more extracellular components are generated by the bacteria. In this stage, bacterial cells are encased by a polymeric matrix and separated from other colonies by water channels (Costerton *et al.*, 1995). Exopolymeric substances interact with inorganic and organic molecules in the environment, such as host-derived proteins (eg: fibrinogen, fibronectin and complement system), which increases the heterogeneity of the biofilm (Dunne, 2002). The growth of any bacterial biofilm is controlled via a quorum sensing system (Maddula *et al.*, 2006), which is affected by the availability of nutrients, optimum hydrodynamic flow, oxygen perfusion, pH, carbon source and osmolarity (O'Toole and Kolter, 1998b). A double mutant strain of *P. aeruginosa*, which could not make quorum sensing signals, was able to adhere and proliferate on a glass surface. When its biofilm was compared to the wild type biofilm, the biofilm of the mutant bacteria was thin and the cells packed densely with no water channels separating them. This indicates that quorum sensing is involved in biofilm differentiation and maturation rather than early attachment (Davies *et al.*, 1998). Several strains of *E. coli* produced tall mushroom like biofilm structures with pores and channels under aerobic

conditions, while the biofilms produced under anaerobic conditions were thinner and less heterogeneous (Bjergbaek *et al.*, 2007).

4) Detachment of planktonic cells from biofilms

It is known that at a certain point, dispersal of bacterial cells from biofilms occurs where the released planktonic cells are free to colonize other surfaces (Costerton *et al.*, 1999). This dynamic process of biofilm dispersal explains the high rate of metastatic infections caused by *S. aureus* (Fatkenheuer *et al.*, 2002). The detached biomass ranges from single cells to 500µm aggregates (Stoodley *et al.*, 2001). Two mechanisms are proposed for the escape of cells from biofilm (Figure 3.4). The first mechanism postulates a physical detachment pathway. When biofilm reaches critical mass, the turbulence and bulk fluid flow takes away fragments of microcolony or planktonic cells that are generated by the outermost layer of the biofilm (Dunne, 2002). In addition, it has been reported that increases in turbulence in the medium surrounding mature biofilm beyond that present during biofilm formation, would result in sloughing of biofilm masses (Simoes *et al.*, 2007). The other mechanism suggests the presence of programmed events leading to local breakdown of the biofilm and conversion of a subpopulation of sessile cells into motile ones. This breakdown is believed to be cell density dependent and regulated by cell-cell signalling (Costerton *et al.*, 1999, Dunne, 2002). A study on *Pseudomonas fluorescens* has shown the involvement of enzymes in the detachment of cells from biofilm exopolymers. The same effect has been noticed after addition of quorum sensing molecules (Allison *et al.*, 1998). Some researchers believe that sloughing of biofilm could be a result of starvation. The response of bacteria to starvation is by degrading exopolysaccharide for consumption and to free cells to seek a better

environment (Watnick and Kolter, 2000). A computer model agreed with experimental model in predicting bacterial sloughing from *P. aeruginosa* in which starvation was the triggering factor (Hunt *et al.*, 2004).



Figure 3.4: Biofilm detachment could be physical one due to shear forces or programmed through quorum sensing (adapted from Costerton *et al.*, 1999).

3.5 *S. aureus* and *S. epidermidis* biofilm cultures in CDM-7 and CDM media, respectively

Many *in vitro* laboratory scale models have been developed to study different aspects of bacterial biofilms. One model, is the small scale perfused biofilm fermenter based on syringe membrane filters (Swinnex, Millipore, UK) (Gander and Gilbert, 1997). In this system cells are attached to one side of the filter and the medium is perfused from the other side (Figure 3.5A). Another method to study biofilms is a microtiter plate-based model. Bacterial suspension is inoculated into the wells of the plate and biofilms form on the surface of the wells (Stepanovic *et al.*, 2000). Modified Robbins device (Tyler instruments, Canada) (Figure 3.5B) is based on a flow cell that has 24 detachable coupons on which bacteria adhere and grow into biofilms (Lewis, 2001). Another model which combines shearing force and microtiter plate is the Calgary Biofilm Device (Ceri *et al.*, 1999). In this device (Figure 3.5C), the lid has 96 pegs that sit in grooved tray.

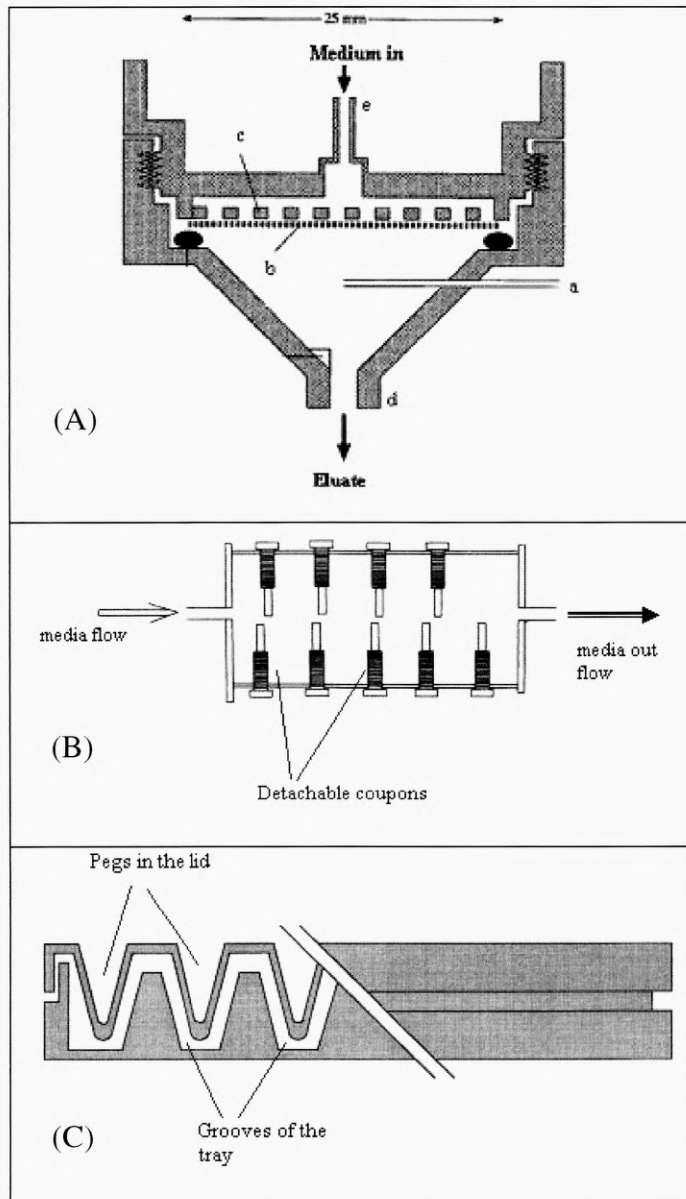


Figure 3.5: Schematic diagrams of different models to study biofilms. Panel (A): small scale perfused film fermenter, cells are loaded on $0.22\ \mu\text{m}$ pore size filter membrane (b) through port (d), membrane is supported by sintered surface (c). The unit is inverted and media introduced through port (e). (a) air vent, (modified from Gander and Gilbert, 1997). **Panel (B):** Modified Robbins device showing media inlet, outlet and the detachable coupons on which biofilms form, (Manz *et al.*, 1993). **Panel (C):** Calgary biofilm device showing the lid containing 96 pegs resting on grooved tray containing media. The whole system sits on tilting device to create shear force (Ceri *et al.*, 1999).

The tray is filled with medium inoculated with cells and placed on a tilting device. The movement will cause the medium to flow creating shear force on the pegs and the formed biofilm. The Sorbarod model (described in Chapter 2) is a continuous flow system based

on a filter plug contained in a tube (Hodgson *et al.*, 1995). The filter is inoculated with bacteria and perfused with medium from one side and the cells shed from the other side. The Sorbarod model was used during this study. It has the advantage over some other biofilm models in that relatively high cell numbers can be recovered from it, which facilitates biochemical studies of biofilm populations and cells released from the biofilm (Hodgson *et al.*, 1995).

3.5.1 Experimental approach

3.5.1.1 *Testing the ability of staphylococci to produce polysaccharide intercellular adhesin (PIA)*

As was mentioned in Chapter 1, PIA or polymeric N-acetyl glucosamine (PNAG) or slime is the exopolysaccharide matrix produced by *S. aureus* and *S. epidermidis* (Cramton *et al.*, 2001; Mack *et al.*, 2004; Higashi and Sullam, 2006). PIA has been found to play an important role in biofilm formation. It is synthesized by *intercellular adhesin (icaADBC)*-encoded proteins (Cramton *et al.*, 1999). PIA mediates bacterial accumulation into multilayer in the second stage of biofilm formation (Higashi and Sullam, 2006) and is implicated in bacterial colonization (Baselga *et al.*, 1993). PIA vaccine protected mice from kidney infection and death from *S. aureus* strains that produced little PIA (McKenney *et al.*, 1999). One of the techniques that has been used to detect the ability of staphylococci to produce biofilm is to test for the production of PIA. Congo red agar method was used widely for this purpose (Baselga *et al.*, 1993; Arciola *et al.*, 2001; Al-Laham *et al.*, 2007; Valle *et al.*, 2007). In this method slime positive strains appear as black colonies with dry crystalline appearance while slime negative strains appear as pink colonies and some times with central dark specks (Chapter 2, Section 2.5.4).

In this project, before growing the bacteria in biofilm systems, they were tested for their ability to produce PIA. *S. aureus* ATCC 6538, 27659 and NCTC 11962 and *S. epidermidis* ATCC 12228 were cultured on Congo red agar (procedure details described in Chapter 2), and all of these strains were found to be PIA positive.

3.5.1.2 Growth of biofilm on Sorbarod

A mid exponential phase culture (3mL inoculum) of *S. aureus* ATCC 6538 grown in CDM-7 medium was used to inoculate a sterile Sorbarod filter (pre-wetted with 5mL normal saline) contained in 10cm PVC tube (Figure 2.3, Chapter 2). The Sorbarod was perfused with sterile CDM-7 medium delivered through peristaltic pump at a constant flow rate of 0.4mL min^{-1} . Sorbarod assembly was incubated at 37°C . Since the number of cells eluting from the biofilm indicates the number of cells dividing within the biofilm (Gander *et al.*, 2005), biofilm growth was monitored by collecting the perfusate at regular intervals. The perfusate (eluate) was collected in sterile receivers and number of cells eluting was estimated by performing viable count after subjecting them to serial dilutions. The same experiment was repeated for *S. epidermidis* ATCC 12228, but replacing CDM-7 with CDM alone.

3.5.2 Results and discussion

Figures (3.6A and 3.6B) present biofilm eluate count of *S. aureus* and *S. epidermidis*, respectively. After inoculating the Sorbarod, there was an initial sharp fall in the count of cells due to the shedding of loosely attached cells into the eluted medium. This behaviour was noticed in all trials using this model (Al-Hmoud, 2002; Masadeh, 2005; Gander *et al.*, 2005). The striking behaviour in these two experiments was that as time passed, count loss was continuous indicating that the rate of bacterial detachment

from the filter was faster than bacterial attachment. In addition, the dynamics of these models were not reproducible, as replicate experiments resulted in different rates for cells detachment.

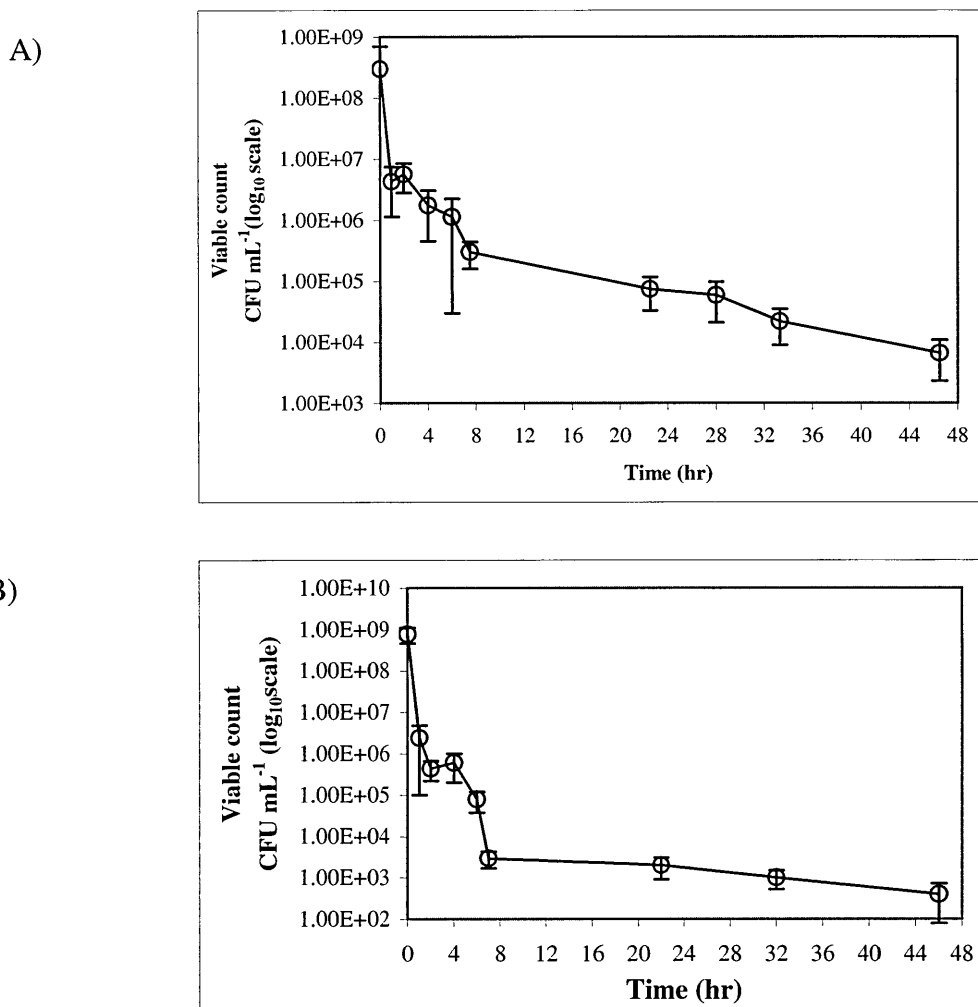


Figure 3.6: Eluate cells count in Sorbarod perfusate with time. (A) *S. aureus* ATCC 6538 in CDM-7 medium. (B) *S. epidermidis* ATCC 12228 in CDM. After the initial sharp loss of loose cells, bacterial loss remained higher than adherent cells and steady state was not achievable. Error bars indicate mean \pm standard deviation of individual data points, $n=3$.

Several attempts were made to change the dynamics of these systems in order to reach steady state, in which bacterial loss and adherent biomass become equal and constant. It is known that during the early stages of biofilm formation on abiotic surfaces, a conditioning film is formed on the surface. The interaction of the surface with its

surrounding environment will result in coating the surface by polymers from the medium (Donlan, 2002). For example, if a foreign material is placed in blood stream, proteinaceous materials, lipids, albumin, inorganic salts and fibronectin will be adsorbed on the surface forming this film (Dunne, 2002). This change in the surface properties of the abiotic surface will increase the affinity and rate of bacteria attaching to it (Donlan, 2002). Moreover, Cabellos-Avelar *et al.* (2006) reported that the use of spent medium from cultures of a strain of *E. coli* promoted the formation of biofilm in another strain, which was unable to form biofilm under anaerobic conditions.

Based on conditioning film concept, two trials were performed in order to improve cell adherence to the Sorbarod. In one trial, an aliquot (5mL) of sterile spent medium was used to pre-wet Sorbarod filter before inoculating it with bacterial culture. Spent medium was prepared by inoculating 25mL of the medium with 250 μ L of overnight culture of the bacteria. The medium was sterilized by filtration (0.2 μ m membrane filter). In another trial, the Sorbarod was wetted with 50% human plasma (diluted in sterile normal saline) before inoculation. In both trials, Sorbarods were perfused with medium for 48hrs and growth was assayed by viable count. In these trials steady state was not achievable and cell detachment was higher than the rate of cells adhering to the Sorbarod. Finally, replacing bacterial strain from *S. aureus* ATCC 6538 with strain ATCC 27659 did not change the dynamics of this system. Since these changes had no positive impact on these biofilm systems in terms of achieving steady state, it was decided to change the medium to a complex medium, eg: brain heart infusion. This medium has been used in many studies to support the growth of staphylococci (Rosten *et al.*, 1987; Sacilik *et al.*, 2001; Miedzobrodzki *et al.*, 2002; Berber *et al.*, 2003).

3.6 Growth curve of *Staphylococcus aureus* in Brain Heart Infusion (BHI)

3.6.1 Preparation of inoculum culture

A sterile Erlenmeyer flask (100mL) containing 25mL of sterile BHI was inoculated with pure culture of *Staphylococcus aureus* NCTC 11962. The flask was incubated in shaking incubator 150 osc min⁻¹ at 37°C overnight.

3.6.2 The test culture

A sterile Erlenmeyer flask (100mL) containing 25mL of fresh BHI medium was inoculated with 250μL of inoculum culture. The flask was incubated in shaking incubator 150 osc min⁻¹ at 37°C. Two test cultures were prepared; one for viable count test, the other for turbidimetric assay, which were performed as previously described (section3. 3, Chapter 3).

3.6.3 Results and discussion

Figure (3.7A) shows the growth curve of *S. aureus* NCTC 11962 in brain heart infusion medium. This medium is a nutrient rich one and has considerably higher amounts of nutrients when compared to CDM or even CDM-7. The lag phase of *S. aureus* in BHI medium lasted for just two hours before the onset of exponential phase. The cells remained in exponential phase for about five hours after which they entered the stationary phase. Mid exponential phase was achieved after 5 hours from inoculation time and the generation time was 31 minutes.

The lag phase of *S. epidermidis* ATCC 12228 was five hours (Figure 3.7 B), after which the bacteria entered in the exponential phase which lasted for seven hours. The generation time (66 minutes) is almost double that of *S. aureus*. Also the stationary phase

was delayed where *S. epidermidis* entered this phase 13 hours after inoculation compared to *S. aureus* (7 hours after inoculation).

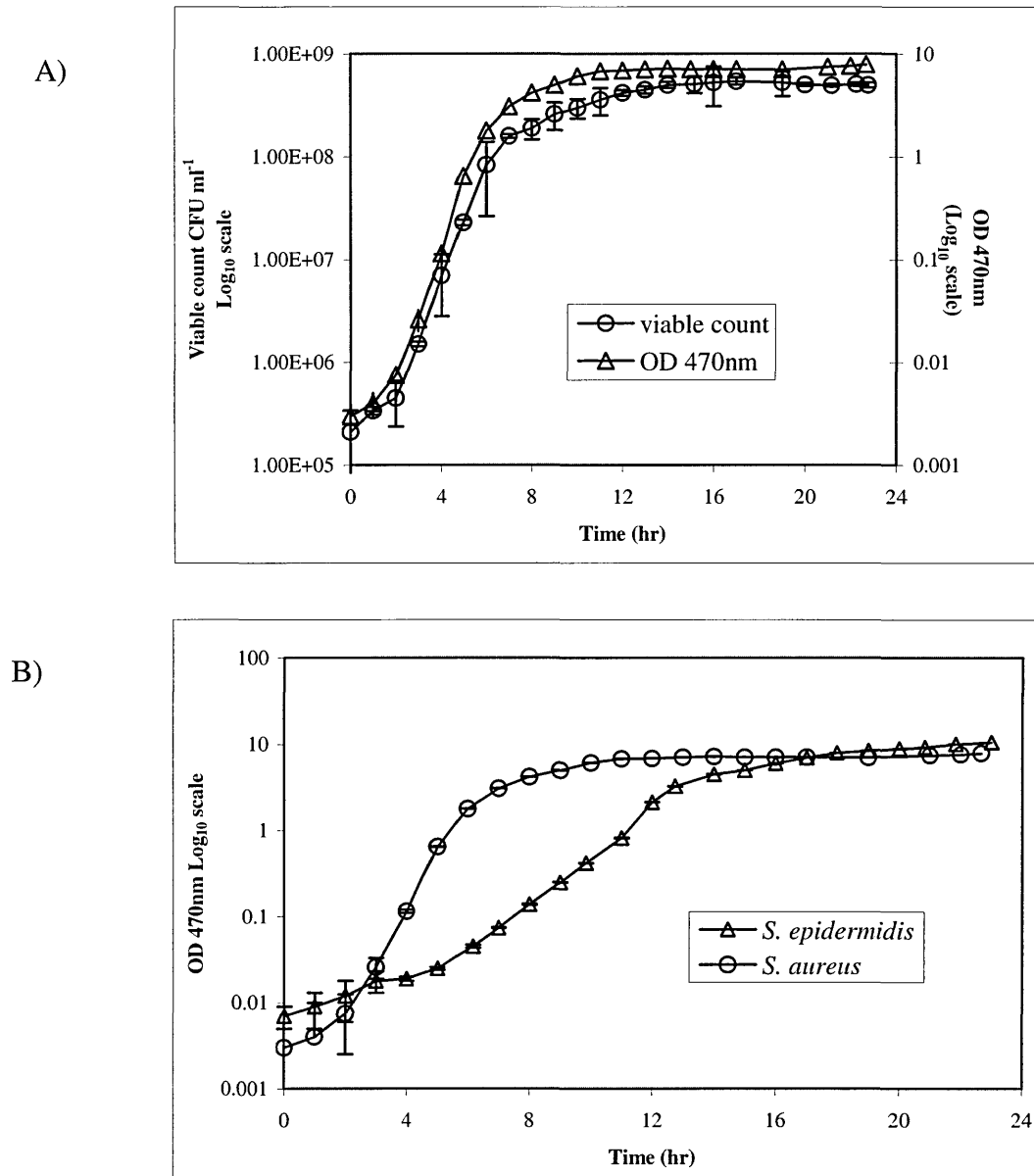


Figure 3.7: *S. aureus* NCTC 11962 growth curve in Brain Heart Infusion medium. (A) mean viable count (log scale) vs time and OD 470nm (log scale) vs time. (B) *S. aureus* growth curve compared to *S. epidermidis* ATCC 12228 growth curve in brain heart infusion medium. Error bars indicate mean \pm standard deviation of individual data points, $n=3$.

3.7 Biofilm cultures of *Staphylococcus aureus* NCTC 11962 in Brain Heart infusion

An aliquot (3mL) of a mid exponential phase culture of *S. aureus* NCTC 11962 grown in BHI medium was used to inoculate (drop wise) a sterile Sorbarod filter (pre-wetted with 5mL normal saline) contained in 10cm PVC tube. The Sorbarod was perfused with sterile BHI medium delivered through peristaltic pump at a constant flow rate of 0.4mL min^{-1} . The Sorbarod assembly was incubated at 37°C . Three Sorbarod units were established and placed in the incubator. To monitor the growth of the biofilm, the perfusate was collected periodically in sterile receivers and assayed for growth by viable count. After 48 hours and after reaching steady state, the Sorbarod filters were removed and either stored at -20°C until required or sacrificed for further studies.

3.7.1 Assay of growth

Aliquots (100 μL) of the eluates were serially diluted in normal saline in the range 10^{-2} to 10^{-8} . Aliquots of the dilutions (100 μL) were spread plated onto brain heart infusion agar plates (pre-dried at 60°C for 5minutes). The plates were prepared in triplicate for each dilution. After 48hr incubation at 37°C , colonies were counted in each plate and viable count was calculated.

3.7.2 Results and discussion

Shortly after inoculation (Figure 3.8) the loosely attached cells were washed out by the flowing medium and the count was reduced by about three log values. Then the loss of cells declined until steady state was achieved where the numbers of detached cells equalled those adhering to the Sorbarod. In this system, steady state was achieved after

11 hours for *S. epidermidis* (Figure 3.8A) and 7 hours for *S. aureus* (Figure 3.8B) from inoculation time.

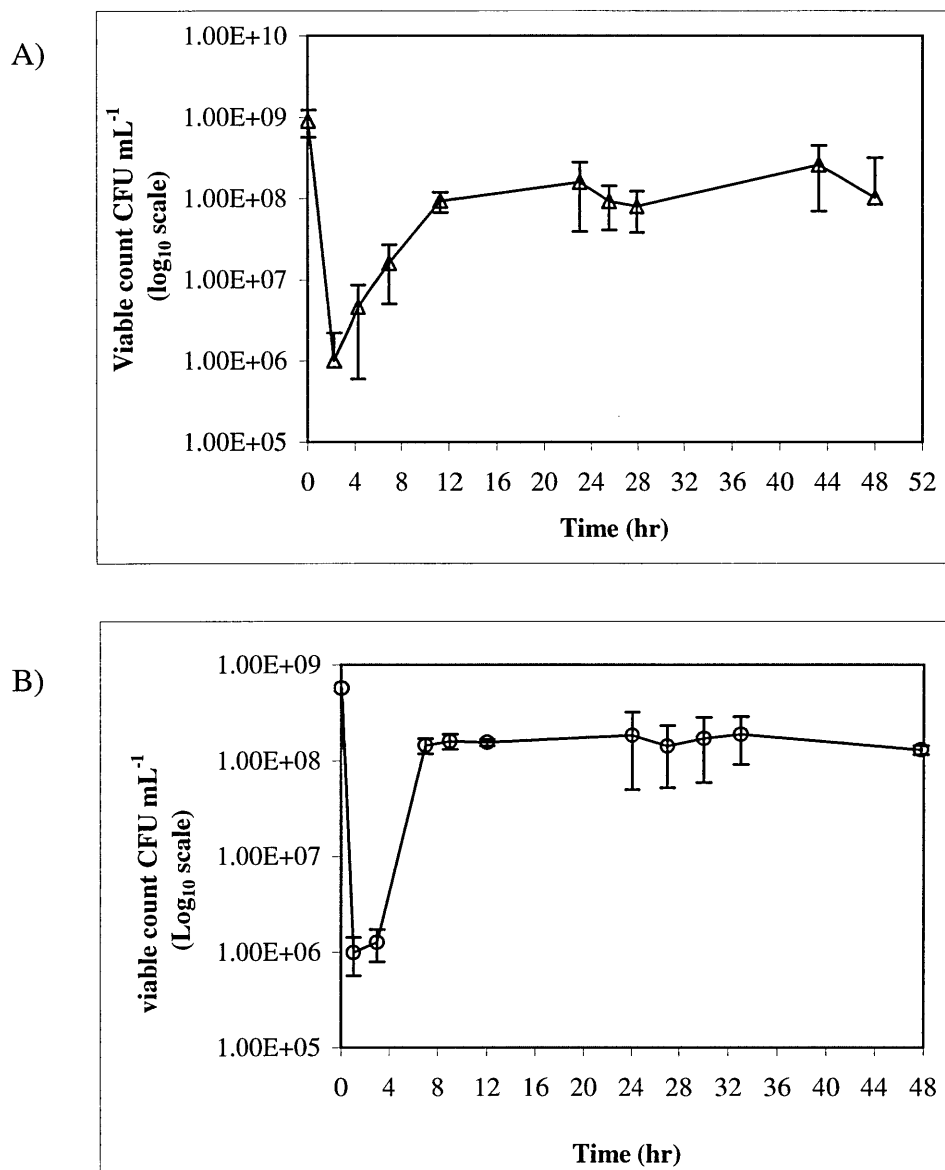


Figure 3.8: Mean biofilm eluate count of (A) *S. epidermidis* ATCC 12228 and (B) *S. aureus* NCTC 11962 in brain heart infusion medium. Each point represents average viable count, n=3 replicates. Error bars represent \pm standard deviation of individual point.

3.8 *In vitro* evaluation of antibiotic activity against biofilm and eluate cultures of *S. aureus* 11962

Standardized methodologies and interpretation that provide reproducible and quantitative results are essential in evaluating antimicrobial agents against bacteria. A generally accepted *in vitro* susceptibility testing is the measurement of minimum inhibitory concentration (MIC; Bloomfield, 1991). By definition MIC is the lowest concentration of antimicrobial agent required to inhibit visible growth of microorganism under certain conditions (Bloomfield, 1991; Andrews, 2001; Curtin and Cormican, 2003). The general principle of MIC determination is to expose a bacterial population (inoculum) to varying concentrations of antimicrobial agent under question, in conditions that are suitable for growth (Curtin and Cormican, 2003). Following an incubation period, the culture medium is inspected for growth. The lowest concentration of antibiotic where no visible growth can be seen in the culture medium is considered to be the MIC (Bloomfield, 1991).

MIC is not a biological constant. Therefore, MIC values are influenced by many factors; the size of inoculum (Svensson *et al.*, 1997), the nature of culture media and incubation period affect MIC values (Bloomfield, 1991; Curtin and Cormican, 2003). The use of higher inoculum density or using enriched media or incubating the culture for longer periods will result in higher MIC values (Bloomfield, 1991; Curtin and Cormican, 2003; Svensson *et al.*, 1997). Thus, MIC values should be compared only if these conditions were standardized. Even with a standardized methodology, a variation within the range of $\pm 1 \log_2$ is considered acceptable for a particular strain (Curtin and Cormican, 2003).

Minimum inhibitory concentration may be determined by several methods. However, two methods are most widely used. These are, (a) the broth dilution method in

which liquid medium is used or (b) the agar dilution method in which solid medium is used. A universally accepted range of antibiotic concentrations to be used for determining MIC is doubling dilution steps up or down from 1000 $\mu\text{g mL}^{-1}$ as required (Andrews, 2001).

In this study, broth dilution (macrodilution method) has been used. In this method, screening to MIC is done by performing two-fold serial dilutions of antibiotic under question in a wide concentration range. The tubes containing the antibiotics are inoculated with a standardized inoculum. After incubation period, MIC is determined. However, it must be remembered that the actual MIC lies between the lowest antibiotic concentration showing no visible growth and the next highest concentration. Once this approximate MIC value is determined, an arithmetic series of dilutions is performed to determine the actual MIC (Bloomfield, 1991).

The aim of this section is to determine minimum inhibitory concentrations (MIC) of selected antibiotics, cefalexin, ciprofloxacin and roxithromycin against biofilm cells and eluate cells of *S. aureus* NCTC 11962. MIC determination will allow selection of suitable sub-MIC of antibiotics to be used to challenge *S. aureus* biofilms. As mentioned earlier in this thesis, the antibiotics were selected from different classes of antibiotics, each one representing a different mechanism of action. The antibiotics used were, (a) Cefalexin (β -lactam; cell wall synthesis inhibitor), (b) ciprofloxacin (fluoroquinolone; interferes with bacterial DNA synthesis) and (c) roxithromycin (macrolide; protein synthesis inhibitor).

3.8.1 Preparation of inoculum

The method described elsewhere was applied after slight modification (Al-Hmoud, 2002; Masadeh, 2005). Three biofilms of *S. aureus* were established on

Sorbarods. They were perfused by brain heart infusion medium (BHI) and incubated at 37°C. After reaching steady state after 48hr, eluate cells were collected into a sterile receiver vessel. The density of bacterial suspension was adjusted to 0.5 McFarland before inoculation.

Sorbarods were sacrificed and disintegrated as described in Chapter 2. The resulting suspension of cells and matrix was decanted into sterile tubes. In order to standardize inoculum size, the density of the suspension was corrected to 0.5 McFarland standard (described in Chapter 2).

3.8.2 Preparation of antibiotic solutions

Cefalexin (potency 93.9%) from Orchid Chemicals and Pharmaceuticals Ltd., India; ciprofloxacin HCl (potency 93.1%) from Dr Reddy's Laboratories Ltd, India and roxithromycin (potency 96.4%) from Alembic Ltd, India, were tested and found to comply with the current European Pharmacopeia .

Stock solutions of antibiotics were prepared in sterile BHI medium to have a final concentration of 1000 $\mu\text{g mL}^{-1}$. The weights of the antibiotics needed to prepare the stock solutions were calculated based on the percentage potency of each antibiotic. Suitable amounts of cefalexin and ciprofloxacin were dissolved separately in BHI medium to prepare 100mL volumes. Due to solubility factors, the required weight of roxithromycin was dissolved first in small volume of sterile dimethylsulfoxide then the volume made up to 100mL with BHI medium. Aliquots (10mL) of the antibiotic stock solutions were distributed in sterile test tubes in triplicate. Double serial dilutions were performed to the amount of stock solutions remaining by adding equal volumes of fresh medium, mixing and distributing 10mL aliquots in other tubes to obtain 500 $\mu\text{g mL}^{-1}$, 250 $\mu\text{g mL}^{-1}$ and so on. Dilutions were prepared to cover the range of 1000 to 0.25 $\mu\text{g mL}^{-1}$.

3.8.3 MIC determination (broth Macrodilution method)

Aliquots (100µL) of biofilm and eluate inocula were added separately to the tubes containing antibiotics. The tubes were mixed by vortexing and incubated at 37°C for 24hrs. After incubation, the tubes were observed for growth and the initial screening MIC value was recorded. The test was repeated where linear dilutions were performed between the concentrations in which growth was seen and absent.

3.8.4 Results and discussion

Table 3.3 shows the results of MIC for cefalexin, ciprofloxacin and roxithromycin against biofilm cells and eluate cells of *S. aureus* NCTC 11962. MIC results of eluate cells when challenged with the antibiotics were the same as those of biofilm cells. Similar results were obtained by Al-Hmoud (2002) when *P. aeruginosa* biofilm cells and eluate cells were tested against benzisothiazolone and zinc pyrithione biocides and the MICs of biofilm cells and eluate cells were found to be equal. Also when the MICs of *Ps. aeruginosa* biofilm cells and eluate cells were determined for cetrimide or sodium pyrithione, MICs of biofilm cells were slightly higher than those of eluate cells (28% and 20% more, respectively). In another study (Masadeh, 2005), the MICs of *Ps. aeruginosa* biofilm cells were two times higher than those of eluate cells when tested against ampicillin, nalidixic acid and streptomycin antibiotics. The same was found when *E. coli* biofilm cells and eluate cells were tested against nalidixic acid and ampicillin. However, the MIC was the same when *E. coli* biofilm cells and eluate cells were challenged with streptomycin (Masadeh, 2005).

MIC results obtained in this study have been used to prepare subinhibitory concentrations of antibiotics in order to investigate their effects on virulence of *S. aureus* biofilms.

Table 3.3: MIC values for biofilm cells and eluate cells of <i>S. aureus</i> NCTC 11962 exposed to antibiotics.		
Antibiotic	MIC ($\mu\text{g mL}^{-1}$)	
	Biofilm cells	Eluate cells
Cefalexin	8	8
Ciprofloxacin	0.8	0.8
Roxithromycin	1	1

3.9 Conclusion

The aim of this chapter was to find a suitable chemically defined medium (CDM) that supports the growth of staphylococci as planktonic and as biofilm structures. A CDM containing the basic nutrients (compound sources of carbon, nitrogen, iron and other ionic sources) was suitable for the growth of *S. epidermidis*, but it was found to give a low *S. aureus* growth yield. Several modifications of the normal CDM formula were studied by step-wise addition of aminoacids until a medium containing seven amino acids (L-valine, L-arginine, L-cystine, L-aspartic acid, glycine, L-leucine and L-proline; named CDM-7), produced the highest growth yield with longer exponential phase and shorter generation time than other media tested. This was tested on three different strains of *S. aureus* and produced similar results. Interestingly, the additional aminoacids gave no effect on growth dynamics of *S. epidermidis*. However, CDM-7 and CDM were not able to support the growth of *S. aureus* and *S. epidermidis* in Sorbarod biofilm models. Therefore, in order to establish biofilm structures on Sorbarods, a complex medium (brain heart infusion broth), was used. After establishing biofilms on Sorbarods, the filters were sacrificed. Biofilm cells and eluate cells were challenged with cefalexin, ciprofloxacin and roxithromycin antibiotics in order to determine their corresponding minimum

inhibitory concentrations. These values will be used to calculate in-use sub-MICs of the antibiotics in order to investigate the effects of these sub-MICs on virulence factors expressed in biofilms.

Chapter 4

Studies of whole cell protein and exoprotein profiles of various cells and supernatants obtained from biofilms of *Staphylococcus aureus* NCTC 11962 challenged with subinhibitory concentration of selected antibiotics

4.1 Introduction

Numerous proteins are expressed by *Staphylococcus aureus*. Some of these proteins are intracellular and others are secreted proteins. The composition and amount of these proteins vary according to the growth phase and external factors. Williams *et al.*, (1999), have shown that cellular proteins of attached *S. aureus* vary with growth phase and that protein content (39 total protein per cell measured by fluorescence) was less than that in planktonic bacteria (74 total protein per cell measured by fluorescence) of the same species and strain. In the late exponential phase to early stationary phase, *S. aureus* makes a switch in protein production from surface proteins to extracellular proteins. This switch is regulated by quorum sensing (Arvidson, 2004). During infection, the bacteria change the expression of their enzymes and proteins to help in tissue invasion and spread (McGavin *et al.*, 1997). Furthermore, when exposed to sub-inhibitory concentrations of antibiotics, staphylococci respond by inducing genotypic and / or phenotypic changes (Gemmell, 1995). These changes will result in reducing the production of some proteins or increasing the production of others (Gemmell, 1995; Stevens *et al.*, 2007). Sub-inhibitory cerulenin has been found to inhibit some staphylococcal exoproteins by blocking their transcription (Adhikari and Novick, 2005). At a concentration of 90% MIC

of linezolid, overall exoprotein secretion of *S. aureus* was reduced (Bernardo *et al.*, 2004). This resulted in significant changes in the exoprotein patterns analysed by SDS-PAGE. When compared to erythromycin, erythromycin induced less evident changes in protein patterns (Bernardo *et al.*, 2004). Toxic Shock Syndrome Toxin 1 (TSST-1) production in seven TSST-1 producing strains was reduced to below detection limit when cells were treated with clindamycin or lincomycin at 1/4 of MIC (Dickgiesser and Wallach, 1987).

Thus, in the following sections, whole cell protein profiles of biofilm cells and eluate cells and exoprotein profile of *S. aureus* NCTC 11962 in the presence of 1/16 MIC of selected antibiotics (cefalexin, ciprofloxacin and roxithromycin) and in their absence (control) were analyzed. The aim was to examine any alterations in protein profiles due to exposure to these antibiotics.

4.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for studying protein profiles.

SDS-PAGE is a widely used technique in biochemistry, genetics and molecular biology to analyse proteins and nucleic acids. Whole cell protein profile obtained by SDS-PAGE analysis has been shown to be able to establish the source of *Salmonella typhimurium*, which can be useful in tracing outbreak strains (Khan *et al.*, 1996). SDS-PAGE analysis of whole cell polypeptide has also been used to identify staphylococcal species (Clinck and Pennington, 1987). However, Pennington *et al.* (1991) were not able to identify coagulase-negative staphylococci by their polypeptide profiles alone. The SDS-PAGE technique has been used widely to identify new proteins (Su and Wong, 1995; De Rossi *et al.*, 1997; Munson *et al.*, 1998) or to track and study changes in exoprotein or cellular protein profiles when the bacteria were exposed to different

conditions (environmental or mutational) (De Rossi *et al.*, 1997; Karlsson and Arvidson, 2002; Bernardo *et al.*, 2004; Adhikari and Novick, 2005; Koszczol *et al.*, 2006). Moreover, SDS-PAGE has been frequently used in following the progress of purification steps of proteins or assessing their purity (Blomster-Hautamaa *et al.*, 1986; Su and Wong, 1995; Munson *et al.*, 1998; Masadeh, 2005; He *et al.*, 2006; Burkhard and Wilks, 2007).

In this technique, proteins are denatured with anionic surfactant, SDS, to be unfolded, linearized and to form soluble negatively charged complex. In general, about 1.4g SDS binds to 1g protein, which will give approximately uniform charge/mass ratio (Smith, 1984). After denaturing the proteins, they are applied to a gel of polyacrylamide and a current is applied where the migration of proteins through the gel is proportional to the size of protein (Smith, 1984). Polyacrylamide gels are formed from the polymerization of acrylamide and N,N'-methylene-bis-acrylamide (a cross-linking agent). The polymerization is initiated by the addition of ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED). The resulting gel is a neutral, hydrophilic, three-dimensional sieve-like matrix of long hydrocarbons cross-linked by methylene groups (Boyer, 2000). Since the proteins after SDS denaturing have the same charge/mass ratio, mobility of the proteins in the gel is a function of pore size of the gel matrix. This mobility is proportional to the molecular weight of the protein. The pore size of a gel depends on the amount of acrylamide and bis-acrylamide present. Higher concentrations give gels with smaller pore sizes allowing the analysis of lower molecular weight biomolecules. In contrast, lower concentrations of acrylamide give gels with large pores allowing analysis of higher molecular weight biomolecules (Boyer, 2000). Laemmli's gels (Laemmli, 1970), are discontinuous gels, that is, they are composed of two different gels (stacking gel and separating gel). These gels differ in their pH and pore size. In stacking gel, all proteins will migrate together in a high speed in the form of a stacked

zone. Once they reach the separating gel, with different gel composition and higher pH, the mobility of proteins will be reduced and separation of proteins based on their molecular weight will commence.

In this project, the gel system used through the study was of slab shape. Slab gels offer the advantage that more than one sample can be analysed at the same time for comparative study. Besides, all samples are analyzed in a matrix and environment that is identical in composition. The buffers used for the gel system were prepared according to Laemmli's method (Laemmli, 1970).

4.3 Experimental approach

4.3.1 Preparation of TSST-1 standard for SDS-PAGE analysis

Toxic Shock Syndrome Toxin 1 (TSST-1; Sigma) powder was reconstituted with distilled water to form 1mg mL^{-1} solution. Equal volumes of this standard and sample buffer (described in Chapter 2) were mixed together and heated at 90°C for 5 minutes. The prepared standard was distributed in small aliquots and stored at -20°C for subsequent use.

4.3.2 Confirmation of TSST-1 production by *Staphylococcus aureus* NCTC 11962 using SDS-PAGE.

In order to confirm the production of TSST-1 from *S. aureus* NCTC 11962, SDS-PAGE analysis of the extracellular proteins produced by these bacteria was done. The extracellular protein profile of a TSST-1 producing strain (NCTC 11962) was compared with the extracellular protein profile of non-producing strain (ATCC 27659) which was used as a control. The membrane-over-agar method was used to assess production of TSST-1 by the bacteria (Parsonnet *et al.*, 1987; Daghistani *et al.*, 2000; Braga *et al.*,

2005; Kansal *et al.*, 2007). In this method, circular sheets of Visking dialysis tubing (Medicell International Ltd, UK) of 12 to 14 kDa molecular weight cut off, were cut to fit Petri dishes. The sheets were sterilized by autoclaving at 121°C, 15psi for 15 minutes. The sterile circles were transferred aseptically and laid on brain heart infusion agar plates. Two plates were prepared, one for each strain. The sheets were inoculated with bacteria by spreading 0.1mL of mid-exponential phase bacterial suspension on the sheets and were incubated at 37°C. After 24hrs, bacterial growth was harvested from the membrane by washing the membrane with 1.5mL sterile normal saline and collecting it in microtubes. The collected suspensions were centrifuged at 13000 rpm (Genfuge 24D, Progen, Moxborough, UK) at 4°C for 15 minutes. Protein concentration in the supernatant solution of each strain was determined by BCA assay (described in Chapter 2). SDS-PAGE analysis was performed for the two strain's supernatants (Chapter 2). Equal amounts of proteins were loaded on previously prepared polyacrylamide gels and constant current was applied using (Mini PROTEAN 3 system, Bio-Rad, Hercules, CA, USA). When the dye front was about to reach the bottom of the gel, the gels were removed and silver stained (Chapter 2).

4.3.2.1 Results and discussion

Figure 4.1 shows exoprotein profile of TSST-1 producing *S. aureus* NCTC 11962 lane 3 and TSST-1 non-producing *S. aureus* ATCC 27659 lane 2. When compared with TSST-1 standard band (molecular weight 22kDa), there is a clear band in lane 3 indicating the presence of TSST-1 in the exoproteins of *S. aureus* NCTC 11962, while in lane 2 no band corresponding to TSST-1 was obtained. These results confirm the production of TSST-1 by *S. aureus* NCTC 11962.

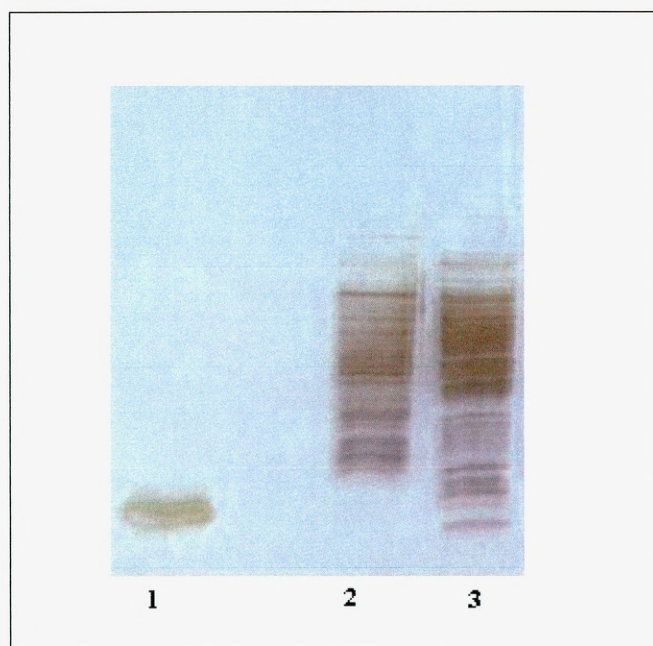


Figure 4.1: SDS-PAGE for exoproteins obtained by membrane-over-agar method. Lane 1: TSST-1 standard (Sigma) ($1\text{ }\mu\text{g well}^{-1}$); lane 2, exoprotein profile of *S. aureus* 27659 non-TSST-1 producing strain; lane 3, exoprotein profile of *S. aureus* NCTC 11962 TSST-1 producing strain. Protein loaded ($8\mu\text{g well}^{-1}$) for sample wells.

4.3.3 Preparing and testing whole cell proteins and exoproteins by SDS-PAGE

Cell pellets of biofilm cells or eluate cells obtained from Sorbarod biofilms exposed to 1/16 MIC of the three antibiotics and a control (prepared as described in Chapter 2), were suspended in a buffer composed of 50mM Tris-HCl buffer containing protease enzymes inhibitors; 1mM Phenylmethanesulphonyl fluoride (PMSF) and 0.5mM N α -Tosyl-L-lysine chloromethyl ketone (TLCK). Protease inhibitors were added to prevent proteolysis and degradation of proteins from protease enzymes that will be released after cell disruption. The volume of the buffer added was twice the volume of the pellet. The cells were disrupted mechanically on ice using an hand held homogeniser (Ultra Turrax T8, S8N-5G, IKA Labortechnik, Stauffer, Germany) as was mentioned in Chapter 2. Cell lysate was distributed in microtubes and clarified by centrifugation at

13000 rpm (Genfuge 24D, Progen, Mexborough, UK) for 15 minutes at 4°C. Protein concentration in the solution was determined by BCA assay (Chapter 2).

For extracellular protein preparation, eluate supernatants were treated with trichloroacetic acid to precipitate the proteins as was mentioned in Chapter 2. After washing the precipitated proteins and drying them under nitrogen, the precipitate was dissolved in buffer (50mM Tris-HCl buffer containing 1mM PMSF and 0.5mM TLCK). Protein concentration was determined by BCA assay. The clear protein solutions obtained from cells or eluate supernatants were mixed with sample buffer (described in Chapter 2) in 1:1 ratio and then heated at 90°C for 5 minutes. After the samples cooled down to room temperature, they were applied into the wells of previously prepared gels using special tips (Gel saver II tips, Fisherbrand, UK). 5 to 20µL of the samples was applied so that equal amounts of proteins were loaded in all sample wells. SDS-PAGE standard marker (3µL) (Serva Electrophoresis, Germany) was applied in a separate well. The gels were run under constant current (30mA) until the dye front reached 2-5mm from the bottom of the gel. The gels were removed and silver stained (Chapter 2).

4.3.4 SDS-PAGE protein markers

The SDS-PAGE protein marker (Serva Electrophoresis GmbH, Heidelberg, Germany) used in this study was ready made, composed of recombinant proteins in the range 10 to 150kDa). The molecular weights (kDa) of the marker proteins were (10, 20, 30, 40, 60, 80, 100, and 150). In order to estimate molecular weights of protein bands in SDS-PAGE gel, protein markers were run with each gel to reduce gel to gel variation. After running the markers, their R_f values were calculated (Table 4.1) and a calibration curve was plotted versus their corresponding molecular weights (Figure 4.2).

4.3.5 Results and discussion

Some distinct variations in protein profiles were observed in biofilm and eluate cells in addition to extracellular proteins when biofilms of *S. aureus* NCTC 11962 were exposed to sub-MIC of cefalexin ($0.5\mu\text{g mL}^{-1}$), ciprofloxacin ($0.05\mu\text{g mL}^{-1}$) and roxithromycin ($0.0625\mu\text{g mL}^{-1}$). Gel analysis has been performed by visually inspecting the gels and comparing protein profiles of treated gels to those of untreated (control biofilms). Repression of some proteins, over / under expression of others or production of new proteins were observed during analysis of the gels. For the purpose of simplicity, only protein bands showing variations from the control were recorded.

Figure 4.3 shows SDS-PAGE gel and Table 4.2 presents summary of findings for treated biofilm cells and control. A new protein (32kDa) was expressed in biofilm cells exposed to cefalexin and roxithromycin but not ciprofloxacin. This protein was not apparent in control biofilm cells. On the other hand, a 25kDa protein which was expressed in control biofilm cells was less expressed in ciprofloxacin treated biofilm cells (appearing as a light band). Similarly, the expression of a 45kDa protein was very low in biofilm cells exposed to roxithromycin when compared to control or other treatments.

Table 4.2: Data analysis of protein profiles obtained from biofilm cells exposed to sub-MIC cefalexin, ciprofloxacin or roxithromycin and control. Protein molecular weights (M_r) were estimated from a calibration curve of protein markers run on the same gel. Data presented here shows proteins on which variations have happened compared to control. P: present; A: absent; N: novel protein.

Protein (M_r) (kDa)	Control	Cefalexin	Ciprofloxacin	Roxithromycin
25	P	P	Light band	P
32	A	P (N)	A	P (N)
45	P	P	P	Light band

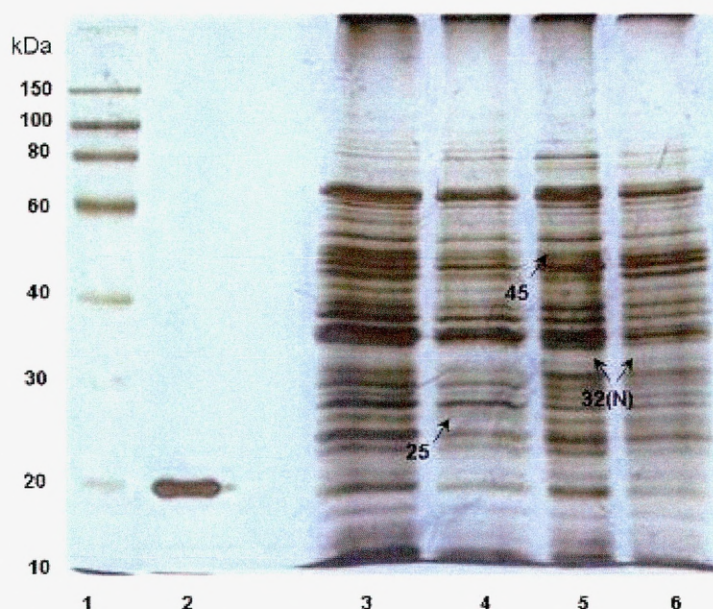


Figure 4.3: SDS-PAGE analysis of biofilm cells. Lane 1, protein markers from 10 to 150kDa; lane 2: TSST-1 standard; lane 3: untreated biofilm cells (control); lane 4: biofilm cells exposed to 1/16 MIC of ciprofloxacin; lane 5: biofilm cells exposed to 1/16 MIC of roxithromycin; lane 6: biofilm cells exposed to 1/16 MIC of cefalexin. Arrows indicate novel protein (N) at 32kDa, light band at 25kDa and light band at 45kDa. (Protein loading $8\mu\text{g well}^{-1}$).

Figure 4.4 and Table 4.3 shows SDS-PAGE analysis for eluate cells exposed to antibiotics and control. A 27kDa protein was suppressed in eluates exposed to sub-MIC roxithromycin, although it was expressed when the cells were exposed to other antibiotics. On the other hand, a 34kDa protein was over expressed by eluate cells exposed to cefalexin. The protein band was darker than those corresponding to the control and the other treatments. Similarly, 82.5kDa proteins were less produced when cells were exposed to cefalexin and roxithromycin, but not ciprofloxacin. Moreover, 95kDa novel protein appeared in cells treated with ciprofloxacin when compared to other treatments and control.

Table 4.3: Data analysis of protein profiles obtained from eluate cells exposed to sub-MIC cefalexin, ciprofloxacin or roxithromycin and control. Protein molecular weights (M_r) were estimated from a calibration curve of protein markers run on the same gel. Data presented here shows proteins on which variations have happened. P: present; A: absent; N: novel protein.					
Protein (kDa)	M_r	Control	Cefalexin	Ciprofloxacin	Roxithromycin
27		P	P	P	A
34		P	P (strong band)	P	P
82.5		P	P (light band)	P	P (light band)
95		A	A	P (N)	A

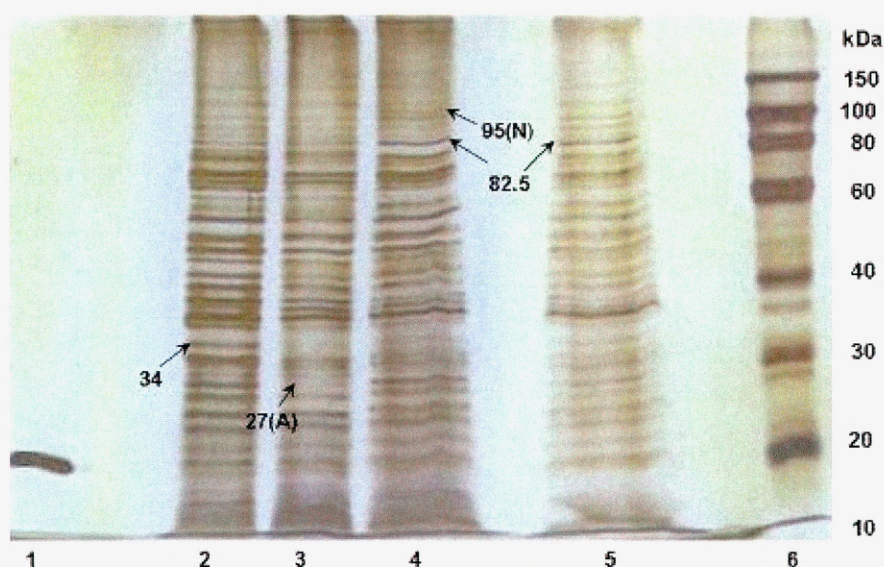


Figure 4.4: SDS-PAGE analysis of eluate cells. Lane 1: TSST-1 standard; lane 2: eluate cells exposed to 1/16 MIC of cefalexin; lane 3: eluate cells exposed to 1/16 MIC of roxithromycin; lane 4: eluate cells exposed to 1/16 MIC of ciprofloxacin; lane 5: untreated eluate cells (control); lane 6: protein markers from 10 to 150kDa. Arrows indicate novel protein (N), absent protein (A), strong band at 34kDa and protein band at 82.5kDa. (Protein loading $8\mu g$ well⁻¹).

Figure 4.5 and Table 4.4 show results of SDS-PAGE analysis for eluate supernatants challenged with antibiotics and control. Two proteins (29kDa and 46kDa) were absent from eluate supernatants of biofilms exposed to cefalexin at sub-MIC level, but were apparent when exposed to ciprofloxacin or roxithromycin and control. On the other hand, a new protein, 28kDa, appeared in the protein profile of cells exposed to cefalexin when compared to the control and other treatments. A 49kDa protein was not

detected in the eluate supernatants when biofilms were perfused with sub-MIC of roxithromycin, but not the other antibiotics. The three treatments caused reduction in the expression of proteins with 22kDa when compared to that of the control.

Table 4.4: Data analysis of protein profiles obtained from eluate supernatants of biofilms which are exposed to sub-MIC cefalexin, ciprofloxacin or roxithromycin and control. Protein molecular weights (M_r) are estimated from a calibration curve of protein markers run on the same gel. Data presented here shows proteins on which variations have happened. P: present; A: absent; N: novel protein.

Protein (M_r)	Control	Cefalexin	Ciprofloxacin	Roxithromycin
22	P	P (light band)	P (light band)	P (light band)
28	A	P (N)	A	A
29	P	A	P	P
46	P	A	P	P
49	P	P	P	A

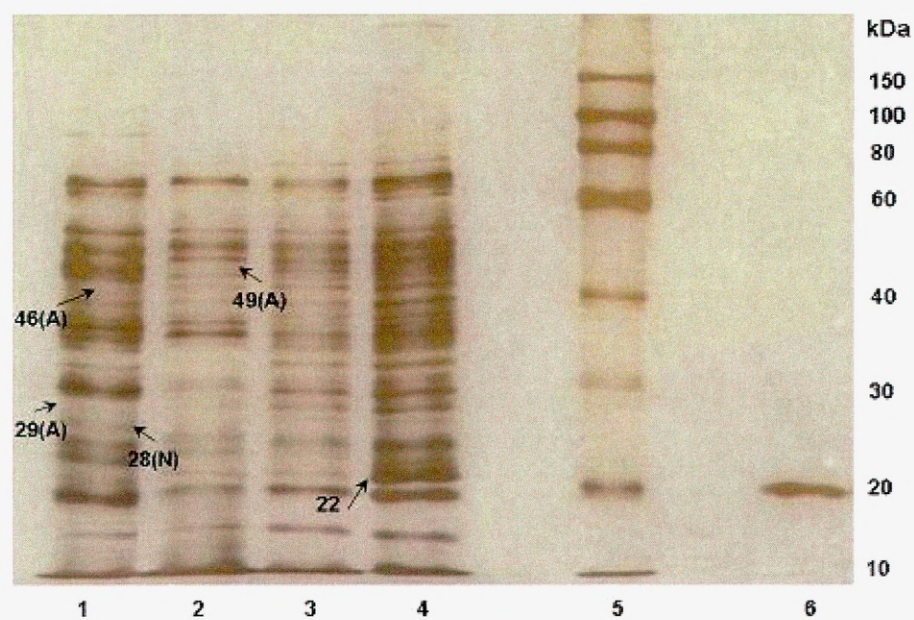


Figure 4.5: SDS-PAGE analysis of eluate supernatants. Lane 1: eluate supernatants exposed to sub-MIC of cefalexin; lane 2: eluate supernatants exposed to sub-MIC of roxithromycin; lane 3: eluate supernatants exposed to sub-MIC of ciprofloxacin; lane 4: untreated eluate supernatants (control); lane 5: protein markers from 10 to 150kDa; lane 6: TSST-1 standard. Arrows indicate novel protein (N), absent protein (A) and strong band at 22kDa. (Protein loading 8μg well⁻¹).

Variations in the pattern of protein profiles of secreted proteins or whole cell proteins obtained from biofilm cells or eluate cells after exposure to subinhibitory concentrations of cefalexin, ciprofloxacin or roxithromycin are apparent. In whole cell protein profiles, some proteins were not expressed in the control, but appeared after exposure to an antibiotic. Other proteins disappeared after exposure to antibiotics. These results indicate that exposure to subinhibitory levels of antibiotics induces series of complex internal reactions in the cell leading to cellular changes. Such changes could be phenotypic, genotypic or in the pathogenicity of the bacterium.

Although SDS-PAGE analysis has earned its reputation as a standard method for protein measurement and analysis, the information it provides is not considered definitive. Since proteins are analysed based on only one dimension or factor (molecular weight), several proteins that share the same molecular weight will migrate at the same rate forming one single band. Thus, for protein identification purposes, other parameters should be considered, such as isoelectric point (by 2D-PAGE analysis) or protein sequencing (mass spectroscopic analysis). Therefore, 2D-PAGE analysis was undertaken for whole cell proteins and exoproteins.

4.4 Two-Dimensional polyacrylamide gel electrophoresis (2D-PAGE)

2D-PAGE is a powerful technique that is used for the separation of proteins in complex mixtures. This method employs separation of denatured proteins according to two different parameters; isoelectric point (pI) by isoelectric focusing in the first dimension, and size by SDS-PAGE in the second dimension. Consequently, this technique can separate thousands of proteins simultaneously and provides information about protein isoelectric point and molecular weight, where each spot on the gel corresponds to a single protein species (Smith, 1984). In the isoelectric focusing step, a

pH gradient is formed in a gel tube or strip by electrophoresis of synthetic polyelectrolytes called ampholytes. Ampholytes are low molecular weight polymers with a wide range of isoelectric points. They are available in different pH ranges (pH 5 to 7, 6 to 8, 3.5 to 10, etc.) (Boyer, 2000).

The net charge on a protein is pH dependent. When the pH is below pI of a protein, the protein will be positively charged and will migrate toward the negatively charged cathode. At a pH above the pI of the protein, it will be negatively charged and will move toward the anode. When the pH is identical to the pI, the protein will have a zero net charge and will not migrate. Thus, when a protein sample is loaded onto a pH gradient strip or tube and a current is applied, proteins in the sample will migrate until each one reaches a region where its net charge is zero, and then it will stop migration. Therefore, when a current is applied, all molecules of a protein located anywhere in the gradient, will migrate toward the region corresponding to their isoelectric point and will stop there, forming a sharp band. In other words, they will 'focus' at their isoelectric point (Boyer, 2000).

In the original technique introduced by O'Farrell in 1975, the first dimension (isoelectric focusing) was performed in narrow tubes of polyacrylamide gel containing carrier ampholyte (O'Farrell, 1975). After setting gel tubes, carrier ampholytes were allowed to migrate throughout the gel by voltage application to form pH gradient. Later, immobilised pH gradient (IPG) strips were introduced in which pH gradients groups were covalently linked to the matrix (Bjellqvist *et al.*, 1982). These immobilized IPG strips overcome the problems encountered with the older carrier ampholyte-generated pH gradients such as pH gradient instability and irreproducibility, in addition to the higher loading capacity and improved resolution (Bjellqvist *et al.*, 1982).

After running the first dimension, the sample gel tube or strip is transferred to a SDS-PAGE slab and electrophoresis is performed in the second dimension to separate proteins based on their molecular weights.

4.4.1 Experimental approach

4.4.1.1 Preparing and testing whole cell proteins and exoproteins by 2D-PAGE

Cell pellets of biofilm cells or eluate cells obtained from Sorbarod biofilms exposed to 1/16 MIC of the three antibiotics and a control (prepared as described in Chapter 2) were suspended in lysis buffer. The volume of lysis buffer added was about twice the pellets' volume. Protease enzymes inhibitors were added to a final concentration of 1mM Phenylmethanesulphonyl fluoride (PMSF) and 0.5mM N α -Tosyl-L-lysine chloromethyl ketone (TLCK). The cells were disrupted mechanically on ice using hand held homogeniser (Ultra Turrax T8, S8N-5G, IKA Labotechnic, Stauffer, Germany) as mentioned in Chapter 2. Cell lysate was distributed in microtubes and clarified by centrifugation at 13000 rpm (Genfuge 24D, Progen, Murbach, UK) for 15 minutes at 4°C. Protein concentration in the solution was determined by BCA assay (Chapter 2).

For extracellular protein preparation, the proteins precipitated from eluate supernatants, which were washed and dried (as described in Chapter 2), were dissolved in about 1mL lysis buffer. Protease inhibitors were added and protein concentration was determined by BCA assay. Aliquots from the prepared samples in lysis buffer were mixed with certain volumes of rehydration buffer (containing DTT and bromophenol blue, described in Chapter 2) so as the final volume of the sample would be 125 μ L. Equal amounts of proteins were prepared for each group of samples under testing. Each sample was used separately to rehydrate IPG strip (Immobiline dry strip pH 3-10NL, 7cm, GE Healthcare, Sweden). After rehydrating IPG strips, the first dimension was run under high

programmable voltage supplied by (Model EPS-3501XL, Amersham Biosciences, Sweden; Chapter 2) and the second dimension was run using (Mini PROTEAN 3 system, Bio-Rad, Hercules, CA, USA; Chapter 2). The gels were silver stained.

4.4.1.2 Results and discussion

The objective of applying SDS-PAGE and 2D-PAGE to whole cell proteins and extracellular proteins, was to detect proteins that might be responsible for any changes in virulence of *S. aureus* biofilms when exposed to sub-MIC of antibiotics. SDS-PAGE analysis of these proteins when biofilms were exposed to cefalexin, ciprofloxacin and roxithromycin has shown that distinct variations in protein profiles occurred as a result of such exposure. Some novel proteins, which were not expressed in untreated cells, appeared in some of the treated ones, others were over-expressed or disappeared in the treated ones. This in turn was reflected on the secreted proteins, which may suggest a role in the expression and activity of secreted virulence factors. Therefore, in order to further investigate these alterations in protein profiles, 2D-PAGE was performed for the three treatments and control.

The estimation of molecular weights and isoelectric points of proteins analysed on 2D-gels were based on calibration curves constructed from two-dimensional electrophoresis protein markers (Figure 2.7 and 2.8, page 54 and 55, Chapter 2). To estimate isoelectric point (pI) for protein spots detected on the gels, Rf value of the first dimension was computed and interpolated on pI calibration curve (Figure 2.7, Chapter 2). Similarly, Rf values of the second dimension were calculated and interpolated on the protein markers calibration curve (molecular weights against Rf values) in order to estimate molecular weights of the protein spots separated on 2D-gels. If the value of calculated Rf was outside the calibration range, extrapolation was performed. Due to

presence of some uncertainty in cases of extrapolation, spots extrapolated were marked to indicate this event.

2D-gels were inspected visually and compared with each other. Protein profiles of control samples (biofilm cells, eluate cells and eluate supernatant) were compared with antibiotic treated corresponding samples. Key proteins that were observed in control samples and treated ones were identified by estimating their pI and molecular weight. Each protein spot was given a number that is unique to this spot, whether it was observed on control gel or treated one. Novel proteins (those that appear in treated sample, but not in the control) or disappearing proteins were identified by estimating their pI and molecular weight. For each treated gel, new proteins were given alphabetical letters for identification. These letters were given serially for each gel, i.e the letter is not unique for each protein.

Figures 4.6, 4.7, 4.8 and 4.9 show 2D-gels for biofilm cells challenged with sub-MIC of antibiotics and control biofilm cells. Tables 4.5, 4.6, 4.7 and 4.8 show estimated molecular weights and isoelectric points of the visualised proteins and their status (novel proteins or disappeared ones). Exposure of *S. aureus* NCTC 11962 biofilms to 1/16 of MIC levels of cefalexin ($0.5\mu\text{g mL}^{-1}$), ciprofloxacin ($0.05\mu\text{g mL}^{-1}$) and roxithromycin ($0.0625\mu\text{g mL}^{-1}$) resulted in several changes in protein profiles of cells and extracellular proteins when compared to those of untreated biofilms (control). Two new proteins with molecular weight 31.5kDa (pI 6.58 and 6.3) (Table 4.6; Figure 4.7, spots F and J respectively) appeared in biofilm cells exposed to cefalexin. These proteins are most likely the same proteins observed in SDS-PAGE gels (protein band at 32kDa) of biofilms treated with same antibiotic (Figure 4.3, lane 6). Similarly, two new proteins with same molecular weight 32kDa and (pI 6.4 and 5.2) (Table 4.8; Figure 4.9, spots E and I) respectively, appeared in 2D-gels of biofilms exposed to roxithromycin.

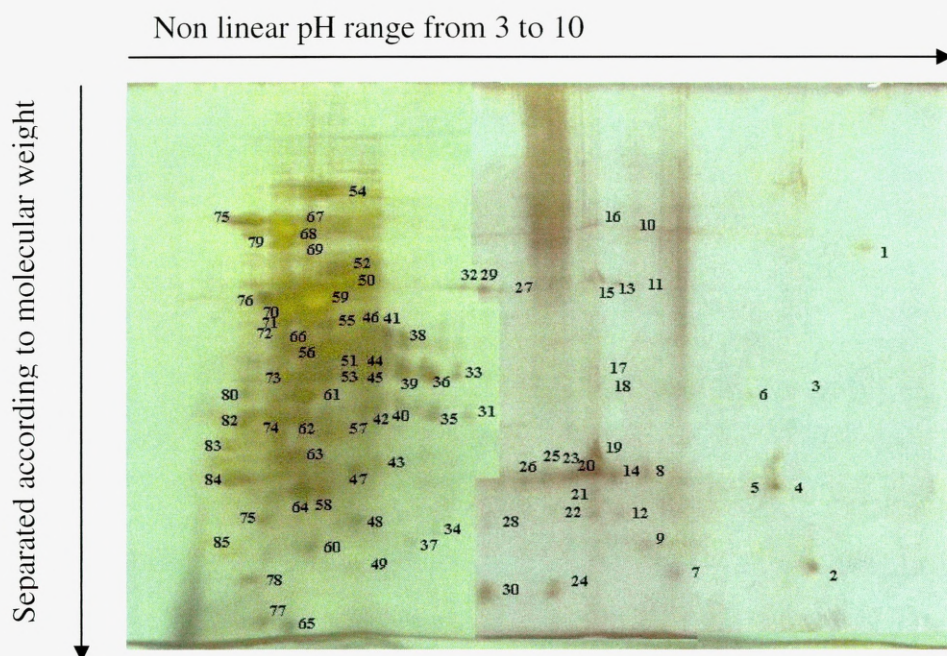


Figure 4.6: 2D-PAGE analysis of untreated biofilm cells (control). Protein spots were numbered for identification (Table 4.5). Protein loading (30µg). This image was combined from two images for clarity in presentation.

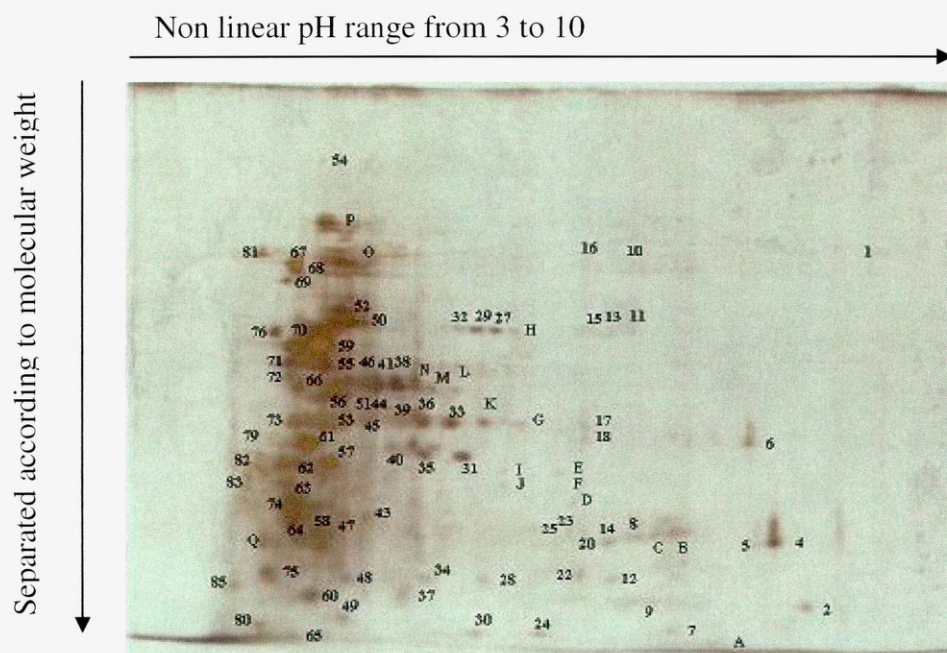


Figure 4.7: 2D-PAGE analysis of biofilm cells exposed to sub-MIC cefalexin. Protein spots were numbered for identification. Missing numbers correspond to missing proteins. Newly appearing proteins were given alphabetical letters (Table 4.6). Protein loading (30µg).

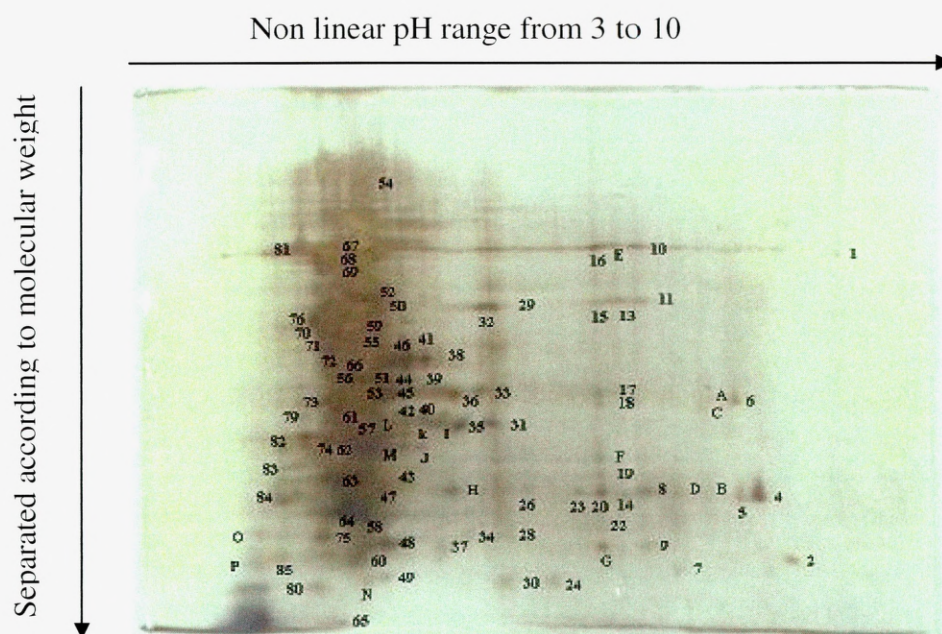


Figure 4.8: 2D-PAGE analysis of biofilm cells exposed to sub-MIC ciprofloxacin. Protein spots were numbered for identification. Missing numbers correspond to missing proteins. Newly appearing proteins were given alphabetical letters (Table 4.7). Protein loading (30µg).

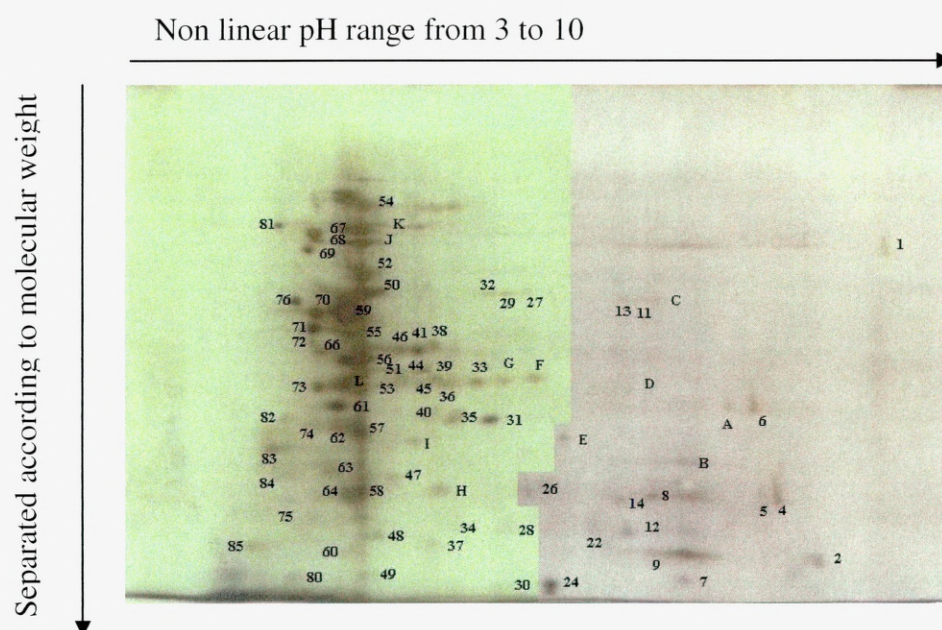


Figure 4.9: 2D-PAGE analysis of biofilm cells exposed to sub-MIC roxithromycin. Protein spots are numbered for identification. Missing numbers correspond to missing proteins. Newly appearing proteins are given alphabetical letters (Table 4.8). Protein loading (30µg). This image was combined from two images for clarity in presentation.

Table 4.5: 2D-PAGE analysis of biofilm cells (control). Spots are identified by a number. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding Rf values. Extrapolated values are marked with (*).

Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
1	85.9	10 *	44	44.5	5.13
2	21.9	9.1 *	45	41.5	5.13
3	43.3	8.9 *	46	51	5.1
4	27	8.6 *	47	28.1	4.96
5	27	8.3 *	48	23.8	4.96
6	41	8.3 *	49	20.5	4.96
7	21	7.54	50	60.4	4.93
8	28	7.26	51	43.5	4.93
9	22.5	7.23	52	62.5	4.85
10	73.4	7.15	53	41.5	4.85
11	61.5	7.15	54	82.8	4.74
12	24.8	7.06	55	51.5	4.72
13	61.5	7.04	56	47.5	4.7
14	28	7.00	57	34	4.7
15	61.5	6.95	58	26	4.7
16	89	6.86	59	56	4.54
17	44.5	6.86	60	21.9	4.65
18	41.9	6.86	61	38.7	4.5
19	29	6.86	62	34	4.5
20	28.3	6.86	63	29.5	4.5
21	26.8	6.86	64	26	4.48
22	24.8	6.86	65	17.8	4.35
23	28.3	6.75	66	50.5	4.3
24	20.3	6.63	67	74.5	4.25
25	28.2	6.56	68	72	4.25
26	27.8	6.43	69	67.4	4.25
27	59.5	6.24	70	55.3	4.25
28	24.5	6.16	71	51.5	4.25
29	58	6.12	72	51	4.25
30	20	6.12	73	41.5	4.25
31	35	5.9	74	34	4.25
32	58.5	5.77	75	24	4.12
33	42	5.73	76	57.5	4.08
34	23.5	5.6	77	18	3.95
35	35	5.57	78	20.4	3.85
36	42	5.53	79	37	3.85
37	26	5.43	80	20.4	3.84
38	48	5.33	81	74.5	3.82
39	43.5	5.33	82	34	3.82
40	36	5.33	83	30.5	3.6 *
41	51	5.25	84	27	3.6 *
42	36	5.2	85	22.4	3.6 *
43	28.5	5.2			

Table 4.6: 2D-PAGE analysis of biofilm cells exposed to sub-MIC of cefalexin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers as they appear in control gel. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding R_f values. Extrapolated values are marked with (*).

Novel Proteins (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	18	7.75 *	3	43.3	8.9 *
B	28	7.45	19	30.8	6.86
C	28	7.28	21	26.8	6.86
D	30.5	6.64	26	27.8	6.43
E	34.3	6.58	42	36	5.2
F	31.5	6.58	77	18	3.95
G	42	6.4	78	20.4	3.85
H	59.5	6.3	84	27	3.5 *
I	34.3	6.3			
J	31.5	6.3			
K	42	6.13			
L	47.5	5.77			
M	47.5	5.6			
N	47.5	5.53			
O	74	4.85			
P	81.5	4.7			
Q	26.5	4.27			

Table 4.7: 2D-PAGE analysis of biofilm cells exposed to sub-MIC of ciprofloxacin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers as they appear in control gel. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding R_f values. Extrapolated values are marked with (*).

Novel Proteins (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	41	8 *	3	43.3	8.9 *
B	28	7.85 *	12	24.8	7.06
C	41.5	7.75 *	21	26.8	6.86
D	28	7.54	25	28.2	6.56
E	73.4	7.04	27	59.5	6.24
F	30.5	6.86	77	18	3.95
G	22.5	6.86	78	20.4	3.85
H	27.8	5.53			
I	35	5.36			
J	30.5	5.3			
K	33.5	5.2			
L	33.5	4.9			
M	30.5	4.9			
N	20	4.5			
O	24.1	3.1 *			
P	22	3.1 *			

Table 4.8: 2D-PAGE analysis of biofilm cells exposed to sub-MIC of roxithromycin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers as they appear in control gel. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding R_f values. Extrapolated values are marked with (*).

Newly appearing spots (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	42	7.65	3	43.3	8.9 *
B	29.5	7.33	10	73.4	7.15
C	61.5	7.04	15	61.5	6.95
D	41.8	6.9	16	89	6.86
E	32	6.4	17	44.5	6.86
F	42	6.3	18	41.9	6.86
G	42	6	19	30.8	6.86
H	27	5.5	20	28.3	6.86
I	32	5.2	21	26.8	6.86
J	72	4.85	23	28.3	6.75
K	75.5	4.8	25	28.2	6.56
L	42	4.6	42	36	5.2
			43	28.5	5.2
			65	17.8	4.35
			77	18	3.95
			78	20.4	3.85
			79	37	3.85

These proteins explain the appearance of new band at 32kDa (Figure 4.3, lane 5) in SDS-PAGE gels for biofilms exposed to similar conditions. A basic protein (pI 8.9, M_r 43.3kDa) disappeared from biofilm cells exposed to any of the three treatments when compared to control (spot 3). Similarly, a 26.8kDa protein (pI 6.86; spot 21; Tables 4.6, 4.7 and 4.8; Figures 4.7, 4.8 and 4.9), which was observed in control biofilm cells, was not detected in the gels of treated biofilm cells. Several neutral proteins (pI range 7.15 to 6.75) disappeared from cells exposed to roxithromycin (spots 10, 15 to 21 and 23) when compared to control. Six protein spots (spots number, 41, 46, 51, 44, 45, 53) corresponding to (51kDa (pI 5.25), 51kDa (pI 5.1), 43.5kDa (pI 4.93), 44.5kDa (pI 5.13), 41.5kDa (pI 5.13), 41.5kDa (pI 4.85), respectively) were observed on 2D-gels of biofilm

cells are more or less, matching those of protein A (minor shifts could be attributed to manual calculations or gel to gel variation). Protein A is a cell-associated protein expressed on *S. aureus* surface and is known to be one of its virulence factors (Mascellino *et al.*, 1989; Jendeberg *et al.*, 1996; Hartleib *et al.*, 2000). In 2D-PAGE analysis, protein A appears as multiple spots (52.6kDa (pI 5.12), 49.2 kDa (pI 4.9), 43.5kDa (pI 4.91), 42.4kDa (pI 5.11), 41.7kDa (5.03), 41.5kDa (4.92)) (<http://www.expasy.org/swiss-2dpage/ac=P99134>). These protein spots were observed in biofilm cells (control) and antibiotic-exposed cells. Some antibiotics have been found to inhibit protein A expression (Gemmell, 1995). However, in this study none of the three antibiotics at 1/16 of MIC was able to inhibit the production of this protein. A protein with M_r 27 kDa and pI 8.6 (spot 4) appearing in 2D-gels is most probably TSST-1 precursor. TSST-1 precursor is known to be produced intracellularly with a molecular weight of 26.3kDa and theoretical pI 8.8 (Blomster-Hautamaa *et al.*, 1986; <http://www.expasy.org>). Before its release, a 40 aminoacid signal peptide is cleaved to give the mature toxin. This protein spot (spot 4) appeared in all biofilm cells (control) and treated ones (Figures 4.6 to 4.9). A spot representing protein with molecular weight of 22.5kDa and isoelectric point 7.23 (spot 9) approximates to TSST-1 (theoretical M_r 22kDa, pI 7.0; Blomster-Hautamaa *et al.*, 1986; Reiser *et al.*, 1983).

Comparison of 2D-PAGE profiles of eluate cells, of control and antibiotic-exposed cells, indicated marked differences among them. Two proteins having the same molecular weight (52.5kDa) (pI 5.77 and 5.68), although absent in control gels (Figure 4.10) were apparent in cefalexin treated cells (Figure 4.11, Table 4.10, spots F and I respectively). Similarly, two new proteins with the same molecular weight (52.5kDa) appeared in gels of roxithromycin treatment. The isoelectric points of these proteins were 6.1 and 5.9 (spots F and H, respectively) (Figure 4.13, Table 4.12). Roxithromycin

resulted in the disappearance of a bright protein spot from eluate cells (spot 34, corresponding to 27kDa, pI 5.25), which was reflected on SDS-PAGE gel of the same cells (Figure 4.4, lane 3). In gels of control and cefalexin treatment, five spots out of six were believed to roughly correspond to those of protein A, (spots 35, 51, 52, 40, 36) which represent (50kDa, pI 5.17; 44kDa, pI 4.85; 41kDa, pI 4.85; 41kDa, pI 5.1; 41kDa, pI 5.17). The sixth spot could be overlapping or underestimated due to the very close molecular weights and isoelectric points of these spots. These spots also appeared in eluate cells of ciprofloxacin and roxithromycin treated cells in addition to the sixth spot of protein A, spot D (48kDa, pI 4.8) in ciprofloxacin gel and spot M (48kDa, pI 4.85) in roxithromycin gel. Spot 2, (26kDa and pI 8.7), which matches that of TSST-1 precursor (26.3kDa, pI 8.8), was observed in all eluate cells whether treated or control. Also spot 14 (22.8kDa and pI 6.95) representing TSST-1, appeared in all gels of eluate supernatants. Several other proteins disappeared and many others appeared due to exposing eluate cells to sub-MIC of antibiotics (Figures 4.11 to 4.13, Tables 4.10 to 4.12).

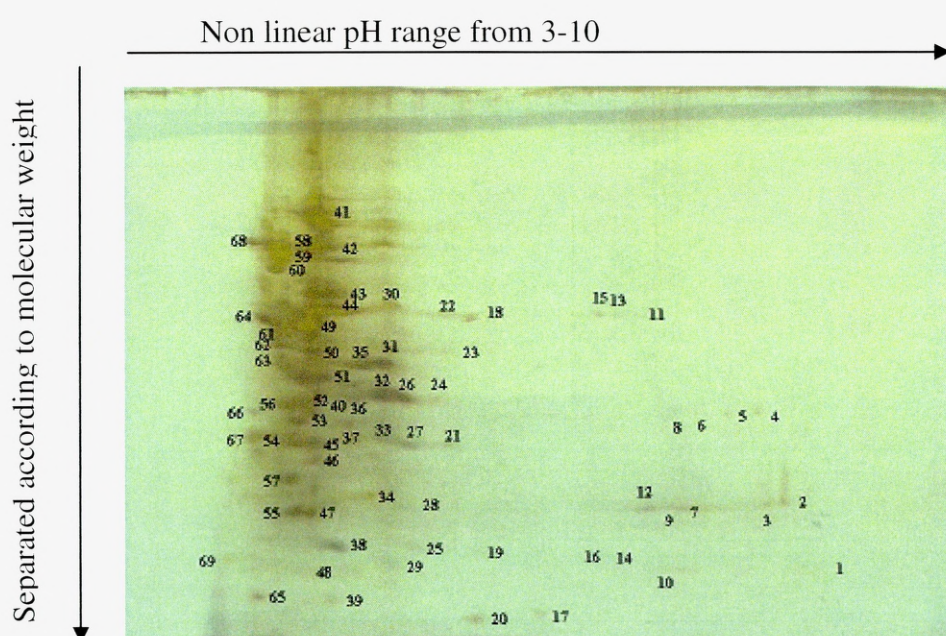


Figure 4.10: 2D-PAGE analysis of untreated eluate cells (control). Protein spots were numbered for identification (Table 4.9). Protein loading (30µg).

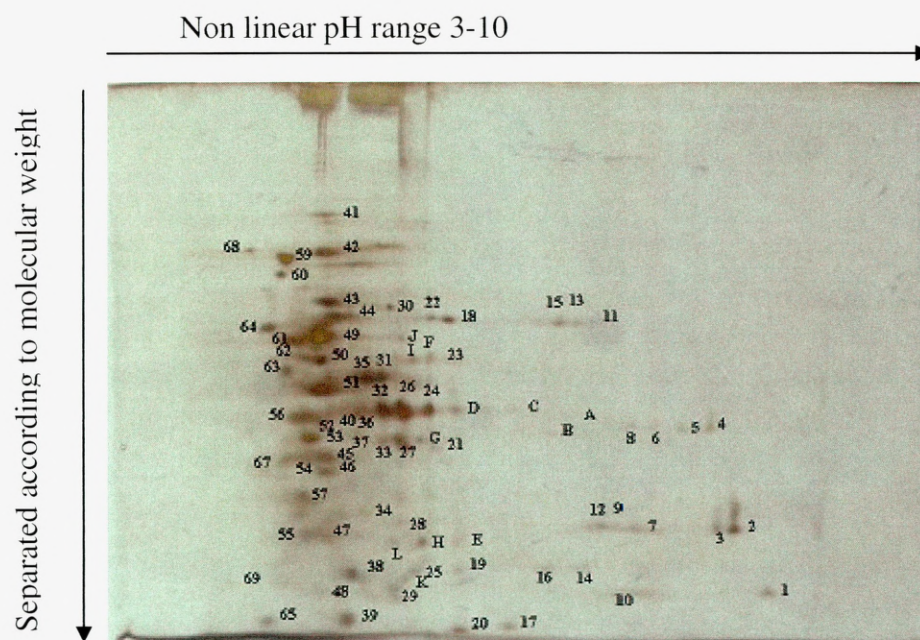


Figure 4.11: 2D-PAGE analysis of eluate cells exposed to sub-MIC cefalexin. Protein spots were numbered for identification. Missing numbers correspond to missing proteins. Novel proteins were given alphabetical numbers (Table 4.10). Protein loading (30µg).

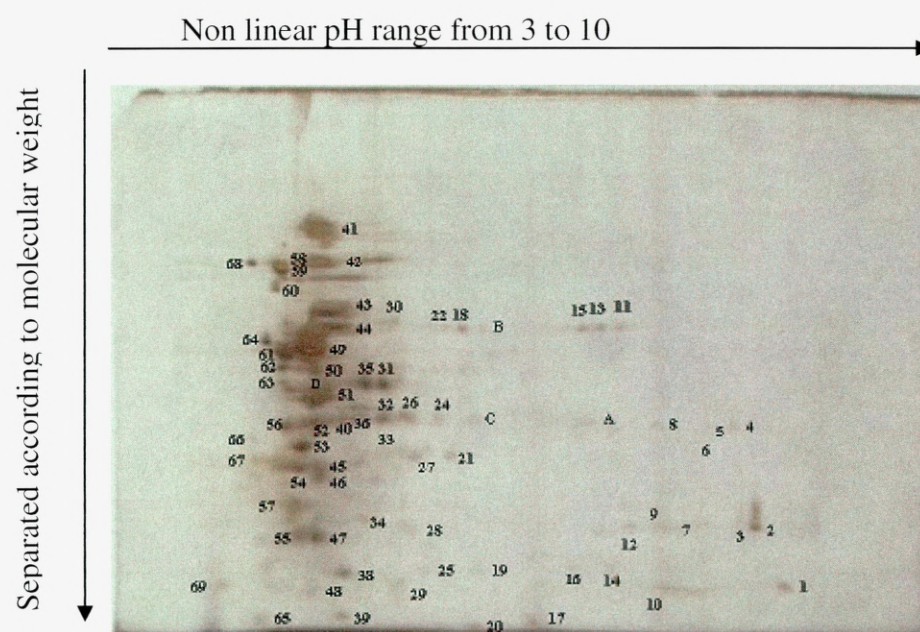


Figure 4.12: 2D-PAGE analysis of eluate cells exposed to sub-MIC ciprofloxacin. Protein spots are numbered for identification. Missing numbers correspond to missing proteins. Novel proteins are given alphabetical numbers (Table 4.11). Protein loading (30µg).

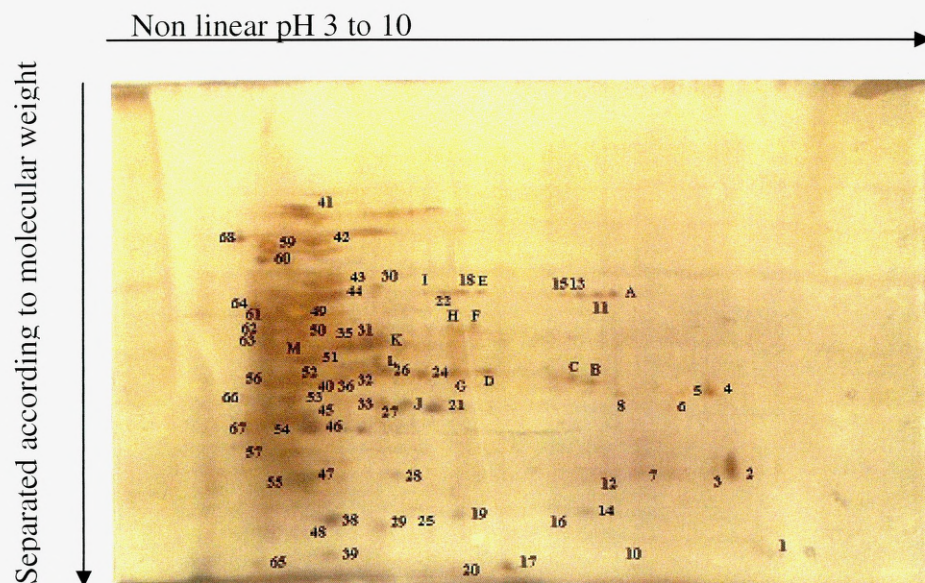


Figure 4.13: 2D-PAGE analysis of eluate cells exposed to sub-MIC roxithromycin. Protein spots are numbered for identification. Missing numbers correspond to missing proteins. Novel proteins are given alphabetical numbers (Table 4.12). Protein loading (30µg)

Table 4.9: 2D-PAGE analysis of eluate cells (control). Spots were identified by a number. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding Rf values. Extrapolated values are marked with (*).

Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
1	18.5	9.3 *	36	41	5.17
2	26	8.7 *	37	35.3	5.12
3	26	8.5 *	38	22.8	5.1
4	38.5	8.4 *	39	19.5	5.1
5	38.5	8 *	40	41	5.1
6	36.3	7.8 *	41	84	5
7	26	7.53	42	72	5
8	38.5	7.44	43	63.8	5
9	26	7.37	44	61	5
10	18.5	7.33	45	31.5	4.9
11	58.5	7.2	46	29.3	4.9
12	26	7.2	47	25.3	4.9
13	58.5	6.95	48	19.5	4.9
14	22.8	6.95	49	57	4.85
15	58.5	6.87	50	51.8	4.85
16	22.8	6.83	51	44	4.85
17	19.3	6.43	52	41	4.85
18	58.5	6.04	53	36.5	4.8
19	22.8	6.04	54	32.5	4.75
20	19	6.04	55	25.3	4.7
21	32.5	5.87	56	39.3	4.52
22	58.5	5.8	57	28	4.52
23	51.8	5.8	58	73	4.43
24	41	5.77	59	72	4.43
25	23.3	5.63	60	69	4.43
26	41	5.57	61	55.5	4.43
27	32.5	5.57	62	55	4.43
28	25.5	5.57	63	50	4.43
29	61	5.48	64	56.5	4.17
30	61.5	5.48	65	20	4.13
31	50	5.4	66	38.5	4.07
32	41	5.4	67	33	4.07
33	32.5	5.4	68	73	4.03
34	27.3	5.25	69	22.5	3.6 *
35	50	5.17			

Table 4.10: 2D-PAGE analysis of eluate cells exposed to sub-MIC of cefalexin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers as they appear in control gel. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding Rf values.					
Newly appearing spots (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	39.5	6.95	58	70.5	4.43
B	33.3	6.86	66	38.5	4.07
C	41.3	6.4			
D	41.3	6.04			
E	24	6.04			
F	52.5	5.77			
G	32.6	5.73			
H	24	5.73			
I	52.5	5.68			
J	56.5	5.58			
K	23	5.58			
L	24	5.53			

Table 4.11: 2D-PAGE analysis of eluate cells exposed to sub-MIC of ciprofloxacin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers as they appear in control gel. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding Rf values.					
Newly appearing spots (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	41.3	6.95	23	51.8	5.8
B	58.6	6.23	37	35.5	5.07
C	41.3	6.04			
D	48	4.8			

Table 4.12: 2D-PAGE analysis of eluate cells exposed to sub-MIC of roxithromycin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers they appear in control gel. Molecular weights (M_r) and isoelectric points (pI) are estimated from their corresponding R_f values. Extrapolated values are marked with (*).

Newly appearing spots (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	58.5	7.46	9	26	7.37
B	36.3	7.13	23	51.8	5.8
C	39.5	6.95	34	27	5.25
D	41.3	6.15	37	35.3	5.07
E	58.5	6.15	58	73	4.43
F	52.5	6.1	69	22.5	3.6 *
G	41.3	5.92			
H	52.5	5.92			
I	58.5	5.76			
J	31	5.6			
K	49.5	5.43			
L	44	5.33			
M	48	4.85			

Exposure of biofilms to subinhibitory concentrations of cefalexin, ciprofloxacin and roxithromycin, resulted in distinct alterations in the pattern of secreted protein profiles, which were consistent with those observed in SDS-PAGE gels. Exposure of biofilms to cefalexin at sub-MIC levels resulted in the disappearance of a 29kDa protein (pI 8.37) from eluate supernatants (spots 5, Figure 4.15, Table 4.14). This protein was present in the supernatants of control biofilms and those exposed to the other treatments. The disappearance of this 29kDa protein was consistent with the disappearance of a band in SDS-PAGE at similar molecular weight (Figure 4.5, lane 1). Moreover, at 46kDa, two protein spots with pIs 5.2 and 5.34 were not apparent when compared to control (spots 22 and 20). The disappearance of these two spots explains the disappearance of a band at 46kDa in SDS-PAGE (Figure 4.5, lane 1). Two new proteins having same molecular weight (28.4kDa, pI 6.4 and 5.32) were secreted / produced when biofilms were exposed

to cefalexin at sub-MIC (spots C and G respectively, Figure 4.15, Table 4.14). These proteins were responsible for the presence of a new band at molecular weight 28kDa in SDS-PAGE gels of supernatants exposed to similar conditions (Figure 4.5, lane 1). A 49kDa protein with isoelectric point 6.25 (spot 13) disappeared from supernatants exposed to sub-MIC of roxithromycin (Figure 4.17, Table 4.16). This protein is the same one which disappeared from SDS-PAGE gel (Figure 4.5, lane 2). Two protein spots (10 and 21) disappeared from supernatants exposed to cefalexin (Figure 4.15, Table 4.14) and ciprofloxacin (Figure 4.16, Table 4.15), but not roxithromycin. A group of alkaline proteins disappeared from supernatants exposed to sub-MIC of cefalexin (pI range 8 to 8.75) (spots 1, 2, 4, 5, 7 and 8) and at the same time, several acidic proteins appeared (pI range 6.09 to 3.75; spots E to R). Several proteins disappeared and new ones appeared in samples treated with antibiotics when compared with control (Tables 4.14, 4.15 and 4.16).). Spot 9 represents TSST-1 (M_r 22kDa and pI 7.25) which is secreted after its cleavage from precursor. This toxin was observed in all 2D-PAGE gels of treated and control supernatant preparations.

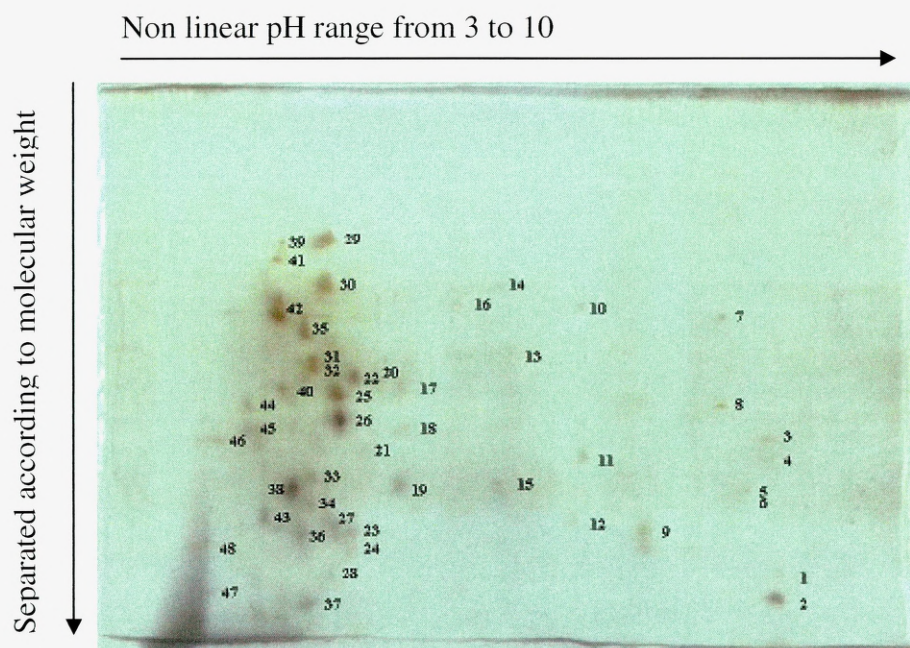


Figure 4.14: 2D-PAGE analysis of untreated eluate supernatants (control). Protein spots are numbered for identification. Protein loading (25µg).

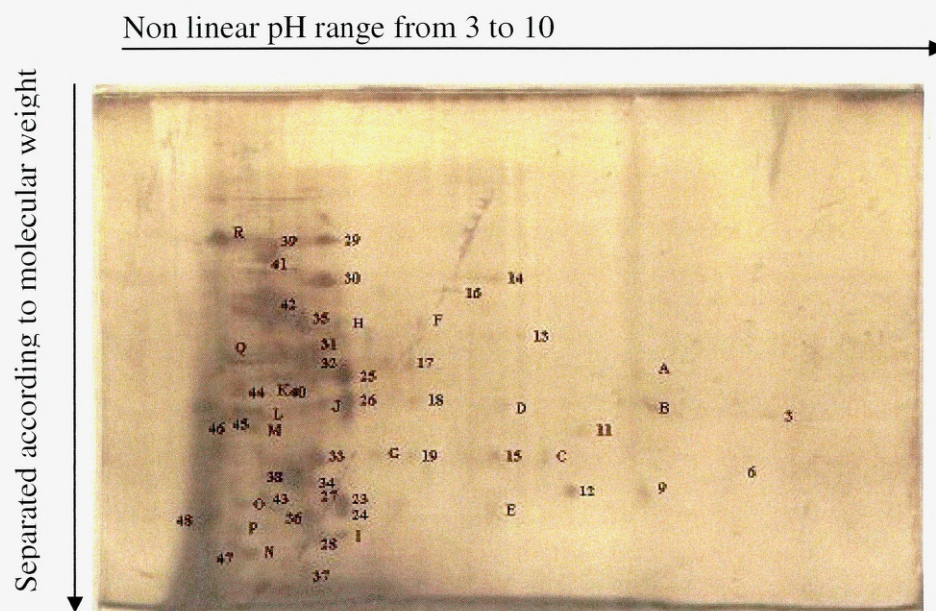


Figure 4.15: 2D-PAGE analysis of eluate supernatants exposed to sub-MIC cefalexin. Protein spots are numbered for identification. Missing numbers correspond to missing proteins. Novel proteins are given alphabetical letters (Table 4.14). Protein loading (25µg).

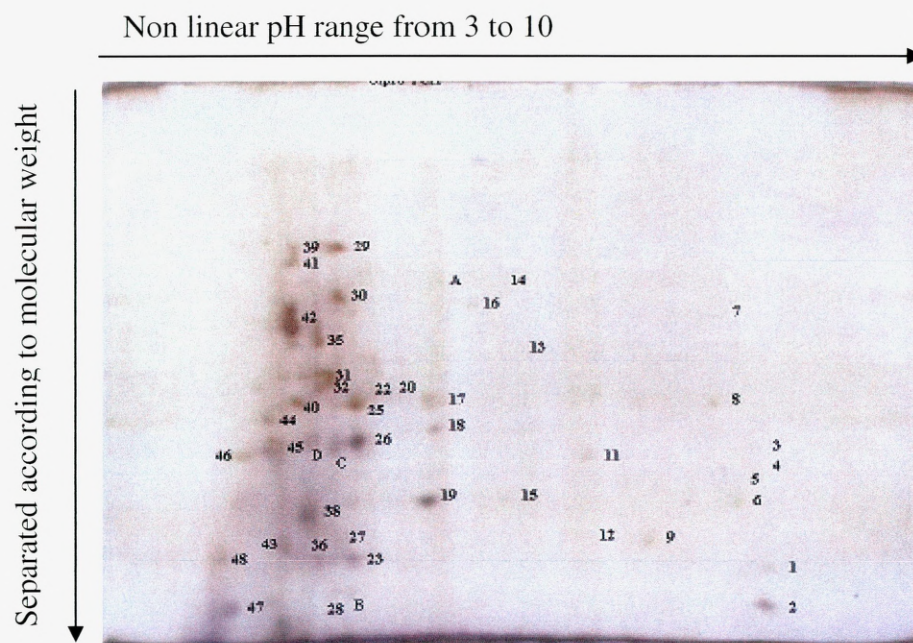


Figure 4.16: 2D-PAGE analysis of eluate supernatants exposed to sub-MIC ciprofloxacin. Protein spots are numbered for identification. Missing numbers correspond to missing proteins. Novel proteins are given alphabetical letters (Table 4.15). Protein loading (25 μ g).

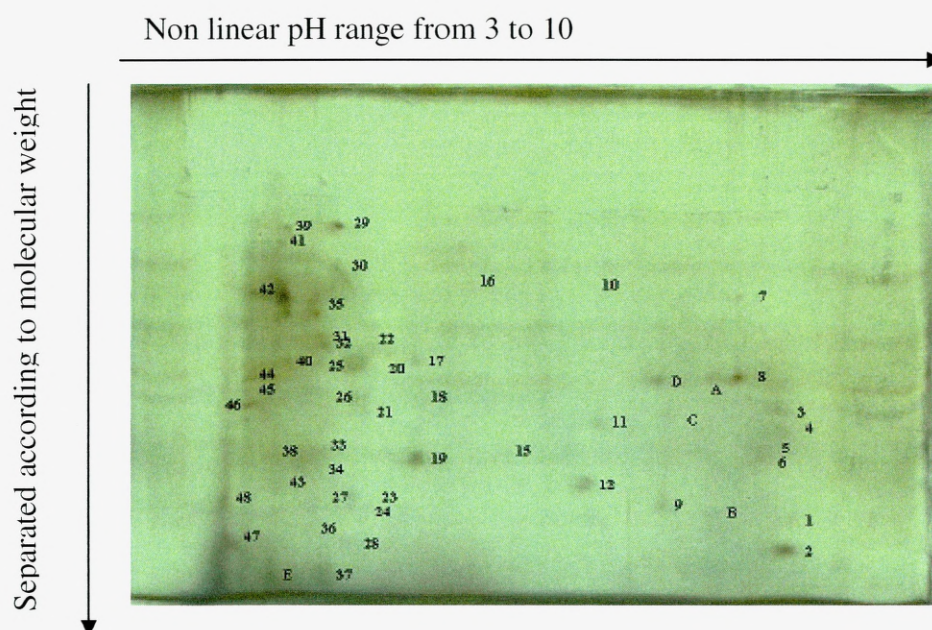


Figure 4.17: 2D-PAGE analysis of eluate supernatants exposed to sub-MIC roxithromycin. Protein spots are numbered for identification. Missing numbers correspond to missing proteins. Novel proteins are given alphabetical letters (Table 4.16). Protein loading (25 μ g).

Table 4.13: 2D-PAGE analysis of eluate supernatant (control). Spots were identified by a number. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding R_f values. Extrapolated values are marked with (*).

Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
1	21.5	8.75*	25	42.5	5.03
2	20	8.65*	26	36	5.03
3	33	8.5 *	27	25	5
4	31	8.5 *	28	20	5
5	29	8.37*	29	74.5	4.94
6	26.5	8.27*	30	64	4.94
7	58.5	8 *	31	49.3	4.84
8	40.6	8 *	32	47	4.84
9	22.8	7.25	33	27.5	4.84
10	60	6.68	34	24.8	4.84
11	31.5	6.68	35	54.8	4.77
12	24	6.54	36	21.2	4.77
13	49.3	6.25	37	17.5	4.77
14	62	6.11	38	26.5	4.70
15	27.5	6.11	39	73.3	4.57
16	59.3	5.8	40	42.5	4.57
17	45	5.51	41	68.4	4.5
18	35	5.51	42	57	4.5
19	27.5	5.48	43	24	4.45
20	46	5.34	44	38.5	4.18
21	32	5.24	45	35.3	4.18
22	46	5.2	46	32	3.97
23	23.5	5.16	47	18	3.75
24	21	5.16	48	21.6	3.75

Table 4.14: 2D-PAGE analysis of eluate supernatants exposed to sub-MIC of cefalexin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers as they appear in control gel. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding R_f values. Extrapolated values are marked with (*).

Newly appearing spots (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	42	7.25	1	21.5	8.75 *
B	34	7.27	2	20	8.65 *
C	28.4	6.4	4	31	8.5 *
D	34.5	6.15	5	29	8.37 *
E	23	6.09	7	58.5	8 *
F	51.7	5.56	8	40.6	8 *
G	28.4	5.32	10	60	6.68
H	51.7	5.05	20	46	5.34
I	21.7	5.05	21	32	5.24
J	36	4.88	22	46	5.2
K	40.8	4.4			
L	33.7	4.3			
M	31	4.21			
N	18	4.15			
O	22.2	4.04			
P	21.4	4.204			
Q	47.5	3.81			
R	75	3.75			

Table 4.15: 2D-PAGE analysis of eluate supernatants exposed to sub-MIC of ciprofloxacin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers as they appear in control gel. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding R_f values.

Newly appearing spots (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	62.5	5.72	10	60	6.68
B	19.8	5.14	21	32	5.24
C	35.3	4.88	24	21	5.16
D	36	4.76	33	27.5	4.84
			34	24.8	4.84
			37	17.5	4.77

Table 4.16: 2D-PAGE analysis of eluate supernatants exposed to sub-MIC of roxithromycin compared to those of control. Newly appearing spots (proteins) are given alphabetical letters. Disappearing spots are given their corresponding numbers as they appear in control gel. Molecular weight (M_r) and isoelectric point (pI) are estimated from their corresponding R_f values. Extrapolated values are marked with (*).

Newly appearing spots (compared to control)			Disappearing spots (compared to control)		
Spot ID	Estimated M_r (kDa)	Estimated pI	Spot ID	Estimated M_r (kDa)	Estimated pI
A	40.6	7.69 *	13	49.3	6.25
B	22	7.69*	14	62	6.11
C	32	7.3			
D	40.6	7.15			
E	19	4.44			

The results obtained from SDS-PAGE and 2D-PAGE analysis showed that marked differences in protein profiles of whole cell proteins and secreted proteins exist among biofilms treated with sub-MIC of different antibiotics on one hand and among treated biofilms and control on the other hand. While many proteins were common between treated and control cells or supernatant samples, others appeared to be novel (proteins that were detected in treated samples, but not in control ones). Moreover, some proteins disappeared from cells or supernatants following exposure to subinhibitory levels of antibiotics.

Since most virulence factors expressed by *S. aureus* (whether cell associated or secreted) are proteins in nature, then the observed alterations in protein profiles due to exposure to sub-MIC of antibiotics may reflect alterations in virulence factor production. It is suggested that this will lead to variations in bacterial pathogenicity. It has been found that protein profile alterations induced by exposure to sub-MIC of antibiotics that act by inhibiting protein synthesis, such as lincosamides and linezolid, were caused by alterations in virulence factors rather than alterations in ribosomal protein or non toxic proteins (Gemmell, 1995; Herbert *et al.*, 2001; Bernardo *et al.*, 2004). One of the reasons

could be that toxin production in *S. aureus* is controlled by membrane associated ribosomes that are more prone to attack by a small number of antibiotics that reach them than cytoplasmic ribosomes, which are concerned with structural and cell maintenance proteins (Gemmell, 1995). The other reason is that, these protein synthesis inhibitor antibiotics act by differential inhibition of the synthesis of one or more of the regulatory proteins (Herbert *et al.*, 2001). This will result in inhibition of synthesis of some virulence factors and the increase in production of the others (Herbert *et al.*, 2001).

Variation in virulence factor production could be a result of alterations at transcriptional level, translational level or alterations in metabolic pathways. For example, subinhibitory concentrations of nafcillin (a β -lactam antibiotic) induced transcription and prolonged mRNA for Panton-Valentine leukocidin, α -toxin and TSST-1 in *S. aureus* (Stevens *et al.*, 2007). In contrast, linezolid and clindamycin repressed translation rather than transcription of those toxin genes (Stevens *et al.*, 2007).

Toxic Shock Syndrome Toxin 1 is a virulence factor secreted by some strains of *S. aureus*. In this study, TSST-1 was observed in order to investigate the effect of subinhibitory concentrations of antibiotics on its production. TSST-1 precursor was observed in biofilm and eluate cells of biofilms exposed to sub-MIC of cefalexin, ciprofloxacin and roxithromycin and controls. Furthermore, TSST-1 was detected in biofilm and eluate cells and eluate supernatants of those treated and control biofilms. The information obtained from electrophoresis indicated the presence of TSST-1 in extracellular secretions of biofilms exposed to the antibiotics, but cannot indicate whether the biological activity of the toxin was altered by exposing biofilms to sub-MIC of antibiotics or not. Therefore, in order to obtain such data, TSST-1 has to be purified from eluate supernatants of biofilms that were challenged with treatments and control, in order to observe its altered biological activity, if any. This study will be discussed in Chapter 6.

In addition, in order to have a better picture of the effect of sub-MIC on secreted virulence factors, these virulence factors should be evaluated individually. Thus, the next chapters will deal with some selected virulence factors.

4.5 Conclusion

The aim of this chapter was to investigate the effect of sub-MIC of cefalexin, ciprofloxacin and roxithromycin on *S. aureus* proteins: whole cell and secreted proteins. Distinct alterations in protein profiles were detected by SDS-PAGE, which were confirmed and more detailed by 2D-PAGE. These shifts in protein profiles are believed to be caused in part by alterations in virulence factor expression whether cell associated or secreted. Hence, selected virulence factors will be further studied to investigate the effect of antibiotic exposure on their production. Furthermore, TSST-1 precursor was detected in the cells exposed to sub-MIC antibiotics and mature TSST-1 was detected in bacterial cells and secretions. Whether the amount of TSST-1 secreted or its biological activity was affected by the exposure to antibiotic or not will be investigated later.

Chapter 5

Effect of subinhibitory concentration of selected antibiotics on some virulence factors of *Staphylococcus aureus* NCTC 11962 biofilms

5.1 Introduction

The pathogenicity of *S. aureus* depends on its ability to produce numerous virulence factors, such as secreted toxins and enzymes, cell wall associated proteins and polysaccharides. These factors may be considered as accessory gene products that are not required for growth and cell division under normal conditions, but to enable microorganisms to adapt to special environmental conditions and to adhere to and invade the host tissues (Herbert *et al.*, 2001).

The clinical efficacy of antibiotics does not depend solely on their ability to produce a bactericidal or bacteriostatic effect on infecting bacteria, but also upon their action on bacterial virulence factor production or release (Bernardo *et al.*, 2004). In general, antibiotics can enhance the release of some virulence factors and down regulate the synthesis and release of others depending on the type of antibiotic (Gemmell, 1995; Stevens *et al.*, 2007). Therefore, the antibiotic-induced regulation of virulence factors could either worsen or attenuate the disease (Bernardo *et al.*, 2004).

As discussed earlier in Chapter 1, the production of staphylococcal virulence factors is regulated through a quorum sensing system. As cell density increases during the post-exponential phase, *S. aureus* switches its virulence factor production from cell associated factors to secreted ones (Ji *et al.*, 1995; Arvidson, 2004). This switch is modulated by global regulators. The most important regulators are the *agr* (accessory gene regulator) and the *sar* (staphylococcal accessory regulator) discussed in Chapter 1.

Several studies have investigated the effect of antibiotics at sub-MIC levels on the production of virulence factors. Sub-MIC nafcillin has been found to induce the production of Pantone leukocidin, toxic shock syndrome toxin 1 and alpha toxin. On the other hand, clindamycin and linezolid significantly suppressed the production of these toxins (Stevens *et al.*, 2007). Sub-MICs of cefodizime down to 1/32 MIC have been found to inhibit the adhesiveness of *E. coli* to human epithelial cells. Also sub-MICs of this antibiotic have been found to reduce haemagglutination and hydrophobicity of *E. coli* (Braga *et al.*, 2000). Sub-MIC linezolid impaired the production of DNase and increased the susceptibility of *Streptococcus pyogenes* to phagocytosis (Gemmell and Ford, 2002). At 1/2 to 1/8 MIC of amikacin and 1/2 to 1/16 MIC of ciprofloxacin, *E. coli* cells changed from hydrophobic state to hydrophilic state and ciprofloxacin at 1/2 MIC was the most effective in reducing the bacterial adherence capability (Wojnicz and Jankowski, 2007).

In this chapter, the effect of exposing *S. aureus* NCTC 11962 biofilms to 1/16 MIC of cefalexin, ciprofloxacin and roxithromycin on the production of coagulase, Toxic Shock Syndrome Toxin 1 and protease will be investigated. In addition, the ability of *S. aureus* to form biofilm in presence of antibiotics at sub-MIC levels will be evaluated.

5.2 Coagulase

Coagulase belongs to a group of proteins that bind specifically to fibrinogen, called the fibrinogen binding proteins (Boden and Flock, 1989; Palma *et al.*, 2001). It is a predominantly extracellular protein that binds to prothrombin forming a complex compound called staphylothrombin that has thrombin-like activity, which converts fibrinogen into fibrin (Kawabata *et al.*, 1986). A fraction of the coagulase is bound to the bacterial cell. This bound form of coagulase was earlier confused with the clumping factor, until it was genetically proved otherwise (McDevitt *et al.*, 1992). Coagulase is

considered one of the most reliable determinants for the identification and classification of staphylococci and it is considered a virulence factor in its own right (Sawai *et al.*, 1997). The role that coagulase plays as a virulence factor appears in some, but not all of the infectious processes induced by *S. aureus*. For example, the *in vitro* adherence of coagulase defective *S. aureus* mutants to either rat fibrinogen or platelet-fibrin clots was not altered when compared to the parental bacteria (Moreillon *et al.*, 1995). At the same time no difference has been noticed in the infectivity between coagulase-deficient mutants and the wild type cells in a rat endocarditis model (Moreillon *et al.*, 1995). In another study, addition of highly purified coagulase to coagulase-deficient mutants of *S. aureus* has no enhancement effect on the virulence of this mutant, while the addition of concentrated culture filtrate of the parent bacteria to the coagulase deficient mutant, enhanced its virulence remarkably (Hasegawa *et al.*, 1983). By studying *S. aureus* Newman strain and its coagulase negative mutant, coagulase has been found to play some role in the attachment of *S. aureus* to purified-fibrinogen-coated surface, but has no significant role in the attachment to plasma-coated surfaces (Dickinson *et al.*, 1995). Meier *et al.*, (2001) transferred the clumping factor gene and coagulase gene to the less virulent bacterium, *Streptococcus gordonii*, which was able to express their gene products. Coagulase positive transformants were neither more adherent to platelet-fibrin clots nor more infective in the rat endocarditis model than the parent, while clumping factor transformants were more adherent and more infective than the parental bacteria. On the other hand, coagulase has been found to play a significant role as a virulence factor in other models. A study in mouse mastitis model has shown that α -hemolysin-negative single mutant and coagulase-negative single mutant have lower virulence compared to wild type (Jonsson *et al.*, 1985). Furthermore, double mutant *S. aureus* has shown a dramatic decrease in virulence, such that only minor changes were observed after

microscopic inspection of the mouse infected tissue (Jonsson *et al.*, 1985). Moreover, in a murine blood-borne staphylococcal pneumonia model, a significant correlation existed between coagulase titre and the number of bacteria recovered from lung tissue (Sawai *et al.*, 1997). When a coagulase-deficient strain was used to infect the lungs, a reduced number of viable bacteria were isolated from the lungs when compared with the parental strain (Sawai *et al.*, 1997). These findings suggest that coagulase is a virulence determinant in *S. aureus*.

Coagulase is mainly synthesized in the exponential growth phase in contrast to other extracellular proteins, which are produced in post-exponential phase. The regulation of coagulase expression is modulated both positively and negatively by the *agr* locus (Lebeau *et al.*, 1994). Many researchers have studied the effect of subinhibitory concentrations of different antibiotics on coagulase as one of the virulence factors. At 1/2 MIC, 1/4 MIC and 1/8 MIC, linezolid (which inhibits bacterial translation) reduced the production of coagulase by 16, 8 and 4 times, respectively, when compared to control, an effect that was attributed to the inhibition of protein synthesis (Gemmell and Ford, 2002). The effect of sub-MIC silver sulphadiazine (a topical antibacterial agent) on coagulase production by two strains of *S. aureus* has been investigated. In one strain, the detection of coagulase in supernatants was delayed for 21 hours with no effect on the amount secreted. However, the production and timing of coagulase in the other strain was not affected (Edwards-Jones and Foster, 2002). No effect of sub-MIC clindamycin has been detected on coagulase production from *S. aureus*, although the production of many other exoproteins was reduced (Herbert *et al.*, 2001).

In this section, the production of coagulase in eluate supernatants of *S. aureus* NCTC11962 biofilms exposed to 1/16 MIC of cefalexin, ciprofloxacin and roxithromycin will be investigated and compared to untreated biofilms.

5.2.1 Experimental approach

5.2.1.1 Preparation of eluate supernatants

Four *S. aureus* biofilms were established on Sorbarods (as described in Chapter 2) at the same time and under exactly the same conditions. The dimensions and weights of Sorbarods were selected to have less than 0.1% relative standard deviation. The filters were inoculated with 3mL of mid-exponential phase of same inoculum culture grown in BHI. In order to avoid minor variations in prepared media composition, the filters were perfused from the same brain heart infusion medium preparation except that, each filter was perfused with BHI containing sub-MIC of one of the selected antibiotics. One filter unit was perfused with BHI without antibiotic, as a control. After reaching steady state, after 48 hours, eluates were collected and supernatants were obtained as was described earlier in Chapter 2. Supernatants were stored immediately at -20°C until required for subsequent analysis.

5.2.1.2 Determination of coagulase titre

In order to determine coagulase activity in a quantitative fashion, the titre of soluble coagulase in culture supernatants was determined. In this method, serial dilutions of supernatants were prepared and mixed with plasma. After incubation, the reciprocal of the highest dilution showing visible plasma coagulation was taken as the titre (Edward-Jones and Foster, 2002; Gemmell and Ford, 2002; Yanagihara *et al.*, 2006).

In this study, eluate supernatants of control biofilm and of biofilms exposed to sub-MIC of cefalexin, ciprofloxacin and roxithromycin were applied in triplicate to 96-well round bottom microtiter plate. The supernatants were serially diluted and equal volumes of citrated rabbit plasma were applied as was described in section 2.9.1 Chapter

2. After 4 hours incubation at 37°C, the plates were visualized against a black background and the titre for each treatment was recorded. The test was repeated three times.

5.2.1.3 Statistical analysis

Statistical difference among the results obtained by the treatments and control were examined by one way analysis of variance (ANOVA), followed by Tukey multiple intergroup comparison. P values <0.05 were considered significant. Since these tests are based on two assumptions, normal distribution of the results and equal variance, these assumptions were checked first. Normality of results was tested using Anderson-Darling test ($p > 0.05$). Equal variance was tested using Bartlett's test ($p > 0.05$). All calculations were carried out using Minitab 15 software.

5.2.2 Results and discussion

Figure 5.1 exhibits the results of coagulase titres for different treatments and Table 5.1 gives the statistical analysis of the results. The results were normally distributed (Anderson-Darling $p = 0.431$, $p > 0.05$) with equal variance (Bartlett's $p = 0.095$, $p > 0.05$). The production of coagulase in eluate supernatants exposed to roxithromycin is the least when compared to other treatments and control. Although it is not significant compared to other treatments, it is significantly less than that of control ($p < 0.05$). This result agrees with the work of others. Moneib *et al.* (1993), who have shown that sub-MICs of new macrolides (azithromycin, clarithromycin and roxithromycin) suppress the production of coagulase from *S. aureus*, although erythromycin has no effect. In another study, protein synthesis inhibitors (chloramphenicol, gentamicin and tetracycline) at sub-MIC levels reduced the production of coagulase (Doss *et al.*, 1993). However, sub-MIC clindamycin had no significant effect on coagulase production in spite of the fact that other

exoproteins were reduced (Herbert *et al.*, 2001). On the other hand, coagulase production in eluate supernatants exposed to sub-MIC ciprofloxacin and cefalexin were not significantly different from each other or from the control (Figure 5.1 and Table 5.1). These results support the results obtained by Doss *et al.*, (1993). In their work, they have found that subinhibitory concentration of methicillin (a β -lactam antibiotic), has no effect on coagulase production, which agrees with the results obtained with cefalexin. Similarly, enoxacin and ciprofloxacin at sub-MIC have been shown not to affect the production of coagulase (Doss *et al.*, 1993). This confirms the result obtained from eluate supernatants exposed to ciprofloxacin.

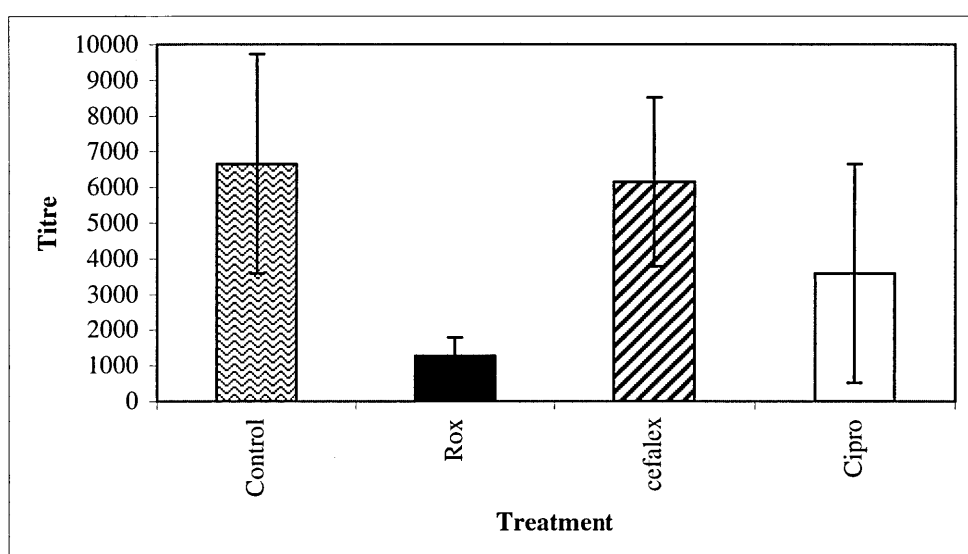


Figure 5.1: Coagulase titre of eluate supernatants exposed to sub-MIC ciprofloxacin (cipro), cefalexin (cefalex), roxithromycin (Rox) and control. Error bars represent standard deviation for each treatment, where n=4.

Table 5.1: Statistical analysis of coagulase titre using Tukey multiple intergroup comparison. Treatments that are significantly different are given different letters. Treatments that are significantly similar are given the same letter.			
Control	Roxithromycin	Cefalexin	Ciprofloxacin
A	B	AB	AB

5.3 Biofilm assay

Biofilm assay or testing the ability of staphylococci to form biofilm *in vitro* has been proposed as a useful marker for the virulence of the bacteria (Davenport *et al.*, 1986; Deighton and Balkau, 1990). In this test, bacteria are allowed to grow in polystyrene microtiter plate wells. After the incubation period, the plate is washed and stained. Optical density of the solubilized stain is measured and is found to be proportional to the amount of adherent biofilms that absorbed the stain.

The biofilm assay has been used widely to detect the ability of bacteria to form biofilm in comparative studies (Djordjevic *et al.*, 2002; Kaplan and Mulks, 2005; Jin *et al.*, 2006) or to quantify the amounts of biofilm produced between different strains or growth conditions (Stepanovic *et al.*, 2000; Stepanovic *et al.*, 2001; Mitrakul *et al.*, 2005; Mohamed *et al.*, 2007). Moreover, the biofilm assay has been shown to be convenient and useful in screening for enteroaggregative *E. coli*, based on the optical density of the stained biofilms (Wakimoto *et al.*, 2004).

In this section, the ability of *S. aureus* to form biofilm in the presence of several subinhibitory concentrations of antibiotics will be quantified and compared with that of control.

5.3.1 Experimental approach

The method used by Stepanovic *et al.* (2000) and their criteria for the evaluation of biofilm formation has been adopted in this study. Serial dilutions of antibiotics in BHI were placed in triplicate in 96-well microtiter plates, so that when the bacterial suspension was added, the concentration of antibiotics cover the range of 90% MIC to 0.35% MIC (procedure details described in Chapter 2, section 2.9.2.2). A positive control was prepared using broth alone without antibiotic. A mid-exponential phase *S. aureus*

culture (adjusted to 0.5 McFarland) was used to inoculate all the wells. Negative controls were prepared by adding broth to eight wells without inoculation. After 24hr incubation at 37°C, the plate was washed and stained (as described in Chapter 2). After solubilizing the dye, the plate was placed on ELISA reader and optical density (562nm) of the wells was recorded.

5.3.2 Data analysis

For comparative analysis of the results, adherence capability of the bacteria was classified into four categories: non-adherent, weakly adherent, moderately adherent and strongly adherent (Stepanovic *et al.*, 2000). Optical density cut-off (ODc) for the microtiter plate test was defined as three standard deviations above the mean OD of the negative control. Table 5.2 shows the classification of adherence capability of the bacteria.

Table 5.2: Classification and criteria of adherence capabilities, OD (optical density), ODc (optical density cutoff) (Stepanovic <i>et al.</i>, 2000)	
Classification	Criteria
Non-adherent	OD less than or equal to ODc
Weakly adherent	OD more than ODc and less than 2 fold ODc
Moderately adherent	OD more than 2 fold ODc and less than 4 fold ODc
Strongly adherent	OD more than 4 fold ODc

5.3.3 Results and discussion

Tables 5.3 and 5.4 show OD results of positive and negative controls and those in the presence of antibiotics, respectively. From Table 5.3, one may observe the OD cut off equals $3 \times 0.0095 + 0.1099 = 0.1384$. Thus, the criteria mentioned in Table 5.2 can be defined and re-written in numerical values (Table 5.5). Accordingly, the ability of *S. aureus* to form biofilm in the absence and presence of sub-MIC of antibiotics is classified

as in Table 5.6. These results show that the ability of untreated bacteria to produce biofilm in microtiter plate is weak. The same was noticed when the bacteria were treated with ciprofloxacin in the range of 90% MIC to 0.35% MIC. This indicates that ciprofloxacin has neither inhibitory nor stimulatory effect on biofilm formation under the experimental conditions applied in this study. Similar results were obtained by Perez-Geraldo *et al.* (1994), when 12 strains of *S. epidermidis* bacteria were exposed to 1/2, 1/4, 1/8 and 1/16 MIC of three fluoroquinolone antibiotics (ciprofloxacin, ofloxacin and sparfloxacin). Compared to control, none of these antibiotics modified bacterial adherence to microtiter plate. Moreover, Rupp and Hamer (1998) reached the same conclusion when sub-MIC ofloxacin, a fluoroquinolone, produced no significant effect on biofilm production of two strains of *S. epidermidis*. When the cells were grown in the presence of roxithromycin in the range of 45% MIC to 0.35% MIC, biofilm formation was similar to that of the control (weak) (Table 5.6). At 90% MIC roxithromycin, the bacteria lost its ability to produce biofilm (Table 5.6). These results agree with the findings of Rachid *et al.*, (2000). In their study, some protein synthesis inhibitors, chloramphenicol, clindamycin and gentamicin produced no effect on *ica* operon expression when used at 1/70 to 1/2 MIC. Therefore, biofilm formation was not affected. On the other hand, other protein synthesis inhibitors, quinupristin-dalfopristin and tetracycline have strongly induced biofilm formation of *S. epidermidis* through enhancing *ica* expression at these sub-MICs (Rachid *et al.*, 2000). The *ica* operon is known to encode the enzymes involved in biosynthesis of polysaccharide intercellular adhesin (PIA) (Heilmann *et al.*, 1996). However, when concentrations above 1/2 MIC were applied, biofilm formation was inhibited (Rachid *et al.*, 2000), which agrees with the observed effect of roxithromycin at 90% MIC.

Cefalexin induced biofilm formation in the range of 2.81% to 45% sub-MIC, while its effect on biofilm formation at lower sub-MIC, 1.41%, 0.7% and 0.35% MIC and at 90% MIC was not significant (Table 5.6). These findings are comparable to those obtained by Dunne (1990). In his study, cefamandole, a cephalosporin antibiotic, resulted in variable biofilm densities when coagulase-negative staphylococci were grown in different sub-MICs in microtiter plates. Twelve percent of the strains exhibited both increased and decreased densities dependant upon the sub-MIC used, 42% of strains had increased biofilm density, while 38% of strains had reduced biofilm density (Dunne, 1990). However, treating two strains of *S. epidermidis* with sub-MIC cefazolin, another cephalosporin drug, resulted in significant decrease in biofilm formation (Rupp and Hamer, 1998).

Table 5.3: OD results at 562nm for positive and negative controls in microtiter plate.		
Parameter	Positive control (OD 562nm)	Negative control (OD 562nm)
Average	0.1532	0.1099
Standard deviation	0.0146	0.0095

Table 5.4: OD results at 562nm for stained biofilms in microtiter plates after being exposed to sub-MICs of different antibiotics.			
% MIC	OD at 562nm, average (standard deviation)		
	Cefalexin	Ciprofloxacin	Roxithromycin
90%	0.211 (0.0227)	0.206 (0.0094)	0.093 (0.0036)
45%	0.431 (0.0159)	0.189 (0.0089)	0.158 (0.0038)
22.5%	0.521 (0.0390)	0.162 (0.0019)	0.189 (0.0148)
11.25%	0.330 (0.0089)	0.149 (0.0079)	0.170 (0.0101)
5.63%	0.310 (0.0146)	0.145 (0.0053)	0.180 (0.0262)
2.81%	0.283 (0.0080)	0.159 (0.0184)	0.174 (0.0170)
1.41%	0.231 (0.0062)	0.139 (0.0084)	0.177 (0.0168)
0.70%	0.223 (0.0040)	0.152 (0.0103)	0.174 (0.0242)
0.35%	0.209 (0.0497)	0.161 (0.0339)	0.176 (0.0233)

Table 5.5: classification and criteria of <i>S. aureus</i> adherence capabilities in numerical values.	
Classification	Criteria
Non-adherent	OD less than or equal 0.1384
Weakly adherent	OD more than 0.1384 and less than 0.2768
Moderately adherent	OD more than 0.2768 and less than 0.5536
Strongly adherent	OD more than 0.5536

Table 5.6: Classification of the ability of <i>S. aureus</i> to form biofilm in microtiter plate based on criteria in Table 5.5.			
% MIC	Biofilm formation classification		
	Cefalexin	Ciprofloxacin	Roxithromycin
90%	Weak	Weak	Non
45%	Moderate	Weak	Weak
22.5%	Moderate	Weak	Weak
11.25%	Moderate	Weak	Weak
5.63%	Moderate	Weak	Weak
2.81%	Moderate	Weak	Weak
1.41%	Weak	Weak	Weak
0.70%	Weak	Weak	Weak
0.35%	Weak	Weak	Weak

5.4 Production of Toxic Shock Syndrome Toxin 1

As was discussed earlier in Chapter 1, TSST-1 is a superantigen that is considered the major causative agent of toxic shock syndrome cases. TSST-1 isolated from toxic shock syndrome patients was isolated from *S. aureus* (Todd *et al.*, 1978; Bohach *et al.*, 1990). Only some strains of *S. aureus* produce TSST-1 (Reeves *et al.*, 1984). Orden *et al.* (1992) have shown that some strains of coagulase-negative staphylococci isolated from mastitis affecting some animals, produced TSST-1. However, Parsonnet *et al.* (1987), observed that none of the 111 coagulase-negative strains isolated from new and used tampons and from the genital tract of toxic shock patients, produced TSST-1. TSST-1 production, as most other *S. aureus* secreted virulence factors, is regulated via *agr* and *sar* loci. TSST-1 production occurs in the post-exponential phase when a critical cell mass is attained (Sarafian and Morse, 1987). However, Wong and Bergdoll (1990), observed that the maximal rate of TSST-1 synthesis was during mid to late-exponential phase. The fact

that TSST-1 production occurs during mid to late exponential phase suggests that nutrient limitation could play a role in its production. Although it is known that nutrient deprived batch culture cells are typical in the stationary phase, several studies have shown that reductions in specific nutrients takes place before the onset of stationary phase (Brown *et al.*, 1990). In general, bacteria respond to nutrient limitation by the so-called stringent response (Godfrey *et al.*, 2002; Jain *et al.*, 2006). Under conditions of nutritional and metabolic stresses, bacteria synthesize an unusual guanosine nucleotide termed GDP 3'-diphosphate or GTP 3'-diphosphate (referred to together as (p)ppGpp or 'magic spot') (Cashel, 1974; Cashel, 1975). The accumulation of (p)ppGpp in the cells will turn on the stringent response. The transcription of stable RNA, such as tRNA and rRNA, will be repressed and the expression of genes involved in the survival of cells under stringent conditions will be upregulated (Chatterji and Ojha, 2001). (p)ppGpp has been found to be involved in various physiological aspects such as quorum sensing, antibiotic production, biofilm formation and virulence (Jain *et al.*, 2006). In one study, biotin limitation resulted in 50 to 200 fold increase of intracellular toxins of *Clostridium difficile* (Karlsson *et al.*, 1999). However, in another study, *Bacillus anthracis* stringent response was responsible for sporulation but not toxin production and virulence (Schaik *et al.*, 2007). Although (p)ppGpp has been found in staphylococci (Cassels *et al.*, 1995), its role in toxin production needs to be elucidated.

On the other hand, *S. aureus* has been found to respond to environmental stress through σ^B factor (described in Chapter 1). However, the role of σ^B factor was found not to be vital for starvation survival (Chan *et al.*, 1998).

Production of TSST-1 is affected by the surrounding environment (Wong and Bergdoll, 1990). Aeration has an enhancing effect on TSST-1 yield up to a limit where excessive aeration has an inhibitory effect. Carbon dioxide has a stimulatory effect on

toxin production (Wong and Bergdoll, 1990). Furthermore, antimicrobials have different effects on the synthesis of TSST-1, depending on the type and concentration of the antibiotic in addition to the bacterial strain (Reeves *et al.*, 1984; Dickgiesser and Wallach, 1987; Gemmell, 1995; Stevens *et al.*, 2007).

Several methods have been used for the detection or quantitation of TSST-1 in culture supernatants or biological media. One of the methods is optimal sensitivity plate (OSP) developed by Robbins *et al.* (1974). It is based on immunodiffusion technique, which is performed in Petri dishes filled with agar of specific type and concentration. The sample containing toxin and antisera specific for the toxin are added separately to wells in the agar. After incubation, lines of precipitation indicate presence of toxin. OSP is used mainly to detect presence of toxin in culture supernatants (Robbins *et al.*, 1974; De Buyser *et al.*, 1987; Daghistani *et al.*, 2000). Enzyme linked immunosorbent assay (ELISA) technique as such or modified have been used widely to detect and quantitate TSST-1 in culture supernatants (Rosten *et al.*, 1987; Daghistani *et al.*, 2000), in vaginal washings (Rosten *et al.*, 1987) and in human serum or plasma (Miwa *et al.*, 1994; Miwa *et al.*, 2000). The principle of one of the ELISA techniques, is to fix specific antibodies to a surface (microtiter wells) and the sample containing the antigens is added so that antigen-antibody interaction occurs. An enzyme conjugated to another antibody is added so that a complex is formed between the antigen and the conjugated antibody. Later, a substrate which can be converted by the enzyme to a detectable, measurable compound is added (Wells *et al.*, 1987; Rosten *et al.*, 1987).

The reversed passive latex agglutination method (RPLA) was first reported as a method for the detection of TSST-1 by Igarashi *et al.* (1986). Before that it was used in Japan for the detection of staphylococcal enterotoxins since it proved to be more sensitive than immunodiffusion method (Igarashi *et al.*, 1986). In this method soluble antigen

(TSST-1 in this case) reacts with its specific antibody that is attached to non-reactive latex particles where the agglutination reaction occurs (Igarashi *et al.*, 1986). Since in standard agglutination, soluble antibody reacts with a particulate antigen, the term (reversed) is used for RPLA. Also, since the latex particles attached to antibody are inert, they are passive, therefore, the method is named Reversed Passive Latex Agglutination.

Takeuchi *et al.* (1998), used RPLA to quantitate the production of TSST-1 from isolates from mastitic cow's milk and farm bulk milk. In addition, RPLA was used to screen for the production of *Staphylococcus* enterotoxins in a food poisoning outbreak (Wei and Chiou, 2002). Moreover, some health authorities use or recommend the use of RPLA test to screen for certain pathogenic toxins (Akhtar, 2005; Feng, 2001).

5.4.1 Detection and quantitation of TSST-1

The amount of TSST-1 present in eluate supernatants of biofilms exposed to sub-MIC antibiotics was determined semiquantitatively by measuring the titre of the toxin in the supernatants (using RPLA kit as was described in Chapter 2), and then multiplying the titre by the detection limit of the anti-TSST-1 antibody.

In order to determine the limit of detection of the antibody suspension in this RPLA kit, it has to be titrated against purified TSST-1 standard (Sigma) with known concentration. To do so, 25 μ L TSST-1 standard (Sigma) (1mg mL⁻¹) was serially double diluted with RPLA diluent in 96-well V-shaped bottom microtiter plate. The concentrations were in the range of 0.25ng mL⁻¹ to 5x10⁵ng mL⁻¹. 25 μ L of latex suspension sensitised with specific rabbit IgG antibody against staphylococcal TSST-1 was added to all dilutions and mixed. The plates were covered and incubated at room temperature on a vibration free surface. After 24hr, the lowest concentration of TSST-1 that produced agglutination was recorded.

5.4.2 Results and discussion

The limit of detection of latex suspension sensitized with specific antibody was found to be 0.5 ng mL^{-1} . Accordingly, the concentration of TSST-1 present in each eluate supernatant was calculated. Table 5.7 shows the results of titre for eluate supernatants exposed to sub-MIC antibiotics and the corresponding concentration of TSST-1.

Table 5.7: TSST-1 titre and concentration in eluate supernatants of biofilms exposed to sub-MIC antibiotics.		
Treatment	Titre	Estimated concentration (ng mL^{-1})
Control	4096	$0.5 \times 4096 = 2048$
Roxithromycin	4096	$0.5 \times 4096 = 2048$
Cefalexin	1024	$0.5 \times 1024 = 512$
Ciprofloxacin	2048	$0.5 \times 2048 = 1024$

The amount of TSST-1 released in supernatants exposed to roxithromycin (protein synthesis inhibitor) is similar to that released in control supernatants, indicating that roxithromycin at 1/16 MIC has no effect on TSST-1 release. Several workers have studied the effect of protein synthesis inhibitors on TSST-1 production. Herbert *et al.*, (2001) found that sub-MIC clindamycin inhibits TSST-1 and other exoprotein synthesis through blocking gene transcription. This blocking is believed to be a result of differential inhibition of the translation of a regulatory protein. In another study (Dickgiesser and Wallach, 1987), 1/4 MIC of clindamycin, erythromycin and lincomycin completely inhibited TSST-1 production. At 1/16 MIC these antibiotics, in addition to kanamycin, significantly but variably reduced TSST-1 production. However, concentrations at 1/16 MIC or lower of tetracycline have no effect on TSST-1 production, although 1/4 MIC reduced its synthesis (Dickgiesser and Wallach, 1987). These results indicate that the effect of protein synthesis inhibitors antibiotics on TSST-1 production depends on the

concentration and type of antibiotic used. This could explain why roxithromycin has no effect on TSST-1 production at 1/16 MIC. Eluate supernatant, which was exposed to ciprofloxacin, produced smaller amounts of TSST-1 than did the control. However, cefalexin resulted in the production of the smallest amount of TSST-1 (Table 5.7). The effect of β -lactams on TSST-1 production is variable. Ampicillin at concentrations up to 10mg L^{-1} has no effect on TSST-1 production (Schlievert and Kelly, 1984). Flucloxacillin at 2 to 10 times MIC was able to inhibit TSST-1 production in the exponential growth phase (van Langevelde *et al.*, 1997). Addition of nafcillin (β -lactam) at sub-MIC levels to mid-exponential phase MRSA and MSSA, prolonged gene transcription of TSST-1. This resulted in prolonged and increased toxin production. However, high concentrations of nafcillin decreased toxin production although mRNA expression remained prolonged (Stevens *et al.*, 2007).

5.5 Proteases

Four types of extracellular proteases are known to be produced by *S. aureus*: these include the serine proteases (V8 protease and related enzyme expressed by Sp1 operon) (Reed *et al.*, 2001), the cysteine protease (Rice *et al.*, 2001), the metalloprotease (aureolysin) (Banbula *et al.*, 1998), and the thiol proteases including protease II and staphopain (Chan and Foster, 1998). These proteases are secreted as proenzymes, which are proteolytically cleaved to produce the mature enzymes (Chan and Foster, 1998; Lindsay and Foster, 1999). Proteases are activated by inter-dependent proteolytic activity. The proform of serine protease is inactive and is cleaved to the active form by a mechanism that involves aureolysin specific process (Drapeau, 1978; Nickerson *et al.*, 2007). The proenzyme of cysteine protease seems to be active and is cleaved by serine protease (Rice *et al.*, 2001).

The production of proteases takes place mainly in the late exponential phase to post exponential phase. Their production is positively regulated by the *agr* locus and down regulated by *sarA* (Lindsay and Foster, 1999).

Staphylococcal proteases perform several functions that contribute to the organisms' pathogenicity. Serine proteases were believed to be potentially involved in neutrophilic inflammation in chronic sinusitis (Sachse *et al.*, 2006). In addition, some proteases have been found to degrade a number of important host proteins, including elastin (Potempa *et al.*, 1988), the heavy chains of all human immunoglobulin classes (Prokesova *et al.*, 1992) and human proteinase inhibitor (Potempa *et al.*, 1986). Moreover, certain proteases have been shown to degrade bacterial surface proteins such as protein A, fibronectin binding protein and clumping factor (Karlsson *et al.*, 2001). Combined, these activities of proteases have been implicated in the spread of the pathogen by a mechanism in which the microorganism becomes less adherent (McGavin *et al.*, 1997). In addition, proteases are thought to down regulate the virulence of *S. aureus* to aid in its colonization in certain *in vivo* niches such as skin and nares (Lindsay and Foster, 1999).

The effect of subinhibitory concentrations of antimicrobials on proteases as part of the virulence factors, has been investigated by several researchers. The inhibitory effect of cerulenin at sub-MIC level on exoprotein production has been found to be at the transcription level. Subinhibitory concentrations of cerulenin have a strong effect on some of the genes encoding serine proteases and on genes encoding α -hemolysin, but have a minimal effect on other genes encoding other serine proteases (Adhikari and Novick, 2005). Subinhibitory concentrations of silver sulphadiazine reduced the production of metalloprotease significantly in one *S. aureus* strain, but not in another

strain. At the same time, total protease secreted remained constant due to the increase in thiol protease and serine protease production (Edward-Jones and Foster, 2002).

In this study, the presence of protease will be detected and quantified by two methods (Zymography and azocasein assay) to investigate the effect of sub-MIC antibiotics on its production.

5.5.1 Experimental approach

5.5.1.1 Zymography

Zymography is an electrophoretic technique to detect for proteolytic activity in polyacrylamide gels. In this technique, developed by Heussen and Dowdle (1980), a large protein substrate such as gelatin or casein is copolymerized with separating gel during casting. The enzymes are applied to migrate and separate under non-reducing denaturing conditions (by sodium dodecyl sulfate). SDS is removed by a detergent, which will refold the enzyme and restore its activity. After incubation in a suitable buffer system, the gels are protein stained to visualise the activity of the enzymes, which is demonstrated by areas free from stain. These areas indicate degradation of the protein substrate by the enzyme. This method has the advantage of detecting proteolytic activity and separating the enzymes based on molecular weight which provides useful qualitative information about the species of the enzyme. In addition, zymography has been used as described or with some modifications to assess proteolytic activity quantitatively (Kleiner and Stetler-Stevenson, 1994; Le *et al.*, 1999; Chu *et al.*, 2004).

In this study, eluate supernatants of the control and treated biofilms were mixed 4:1 with sample buffer (5x) (described in section 2.9.3 in Chapter 2). Proteinase K (1mg mL⁻¹) solution was used as a positive control for proteolytic activity after mixing it with sample buffer (5x). Aliquots (22µL) of the samples and positive control were applied into

the wells of stacking gel (0.75mm). Electrophoresis was performed in mini Protean 3 system (Biorad, USA). After processing the gel (washing and incubation, as described in Chapter 2), it was stained and then destained to visualize proteolytic activity (Chapter 2).

5.5.1.2 Azocasein assay

Azocasein is a chemically modified protein composed of casein (milk protein) attached to orange azo-dye compound, sulphanilamide. Proteolysis releases acid soluble azopeptide resulting in orange-red colour that can be measured spectrophotometrically (Miedzobrodzki *et al.*, 2002). The intensity of the colour is proportional to the activity of proteases. In this method, total proteolytic activity is determined by dissolving the sample containing the enzyme into buffered azocasein solution and incubating it at 37°C for a specific time to allow for the proteolytic reaction to proceed. After stopping the reaction and separating the unreacted protein, the absorbance of the supernatant is measured spectrophotometrically (Brock *et al.*, 1982; Miedzobrodzki *et al.*, 2002). One unit of activity is defined as an increase in the absorbance by 0.001 after 1hr incubation (Edward-Jones and Foster, 2002). Thiol, serine and metalloprotease activity can be determined by the addition of appropriate inhibitors and subtracting the activity from the total one (Edward-Jones and Foster, 2002). Serine protease activity may be inhibited by the addition of phenyl methyl sulphonyl fluoride (PMSF, 12.5mM); thiol protease activity may be inhibited by the addition of iodoacetic acid (12.5mM) and metalloprotease activity may be inhibited by the addition of ethylenediamine tetraacetic acid (EDTA, 12.5mM).

In this work, 50µL of eluate supernatants of control biofilm and those exposed to sub-MIC antibiotics (prepared in section 5.2.1.1) were added to incubation mixture and incubated for 3 hours (procedure details described in Chapter 2). After stopping the

reaction and separating the unreacted protein, the absorbance at 365nm was measured. An aliquot (50µL) of BHI treated in the same way was used as a blank.

5.5.2 Results and discussion

After inspecting the zymogram (data not shown), no colourless bands in the gel corresponding to the treated eluates or control eluates have been observed, indicating the absence of protease activity. A clear colourless band corresponding to the proteinase K (positive control) appeared indicating the activity of the enzyme and the validity of the test. In order to confirm these results, an azocasein assay was conducted. Absorbencies of zero values were obtained for all eluate samples (treated and control biofilms) indicating absence of proteolytic activity (data not shown).

The above results indicate that under the mentioned conditions, protease activity was absent in the eluate supernatants of *S. aureus* NCTC 11962, whether untreated or exposed to sub-MICs of cefalexin, ciprofloxacin or roxithromycin. Similar results have been obtained with other workers. *S. aureus* (67 strains) isolated from skin lesions of patients with atopic dermatitis were shown to exhibit variable proteolytic activities (Miedzobrodzki *et al.*, 2002). Although eleven strains exhibited high activity, six strains gave no detectable activity when tested by azocasein assay (incubation for 3 hours). Similarly, 6 strains exhibited zero activity when tested by a qualitative method (casein agar plate), where the plate was incubated with the sample for 24hr. Interestingly, when the agar plates were incubated with the sample for a longer time (72hr), four strains out of the six strains exhibited proteolytic activity. This indicates that the non-active strains could have proteolytic activity that is below the detection threshold (Miedzobrodzki *et al.*, 2002). In another study (Karlsson and Arvidson, 2002), 92 strains from infected human soft tissue were tested for proteolytic activity. Twenty one strains produced

proteolysis on casein agar plates, and the remaining 71 strains appeared to be protease-negative. Some strains from the protease-negative and protease-positive ones were screened for protease genes. All the screened strains were found to contain the major protease genes. A significant correlation was noticed between the level of protease mRNA and the zone of proteolysis on casein agar plates. In addition, an inverse correlation has been observed between the levels of *sarA* mRNA and protease mRNA. Further analysis has shown that protease genes are intact and their expression is down regulated in most *S. aureus* clinical strains. This was attributed to high σ^B -dependent *sarA* at least under specific *in vitro* growth conditions (Karlsson and Arvidson, 2002).

Therefore, the absence of proteolytic activity in *S. aureus* NCTC 11962 under the specified conditions, could be a consequence of elevated level of protease gene repressors that were not affected or down regulated by the presence of antibiotics.

5.6 Conclusion

The exposure of *S. aureus* NCTC 11962 to sub-MICs of antibiotics resulted in some moderation of its normal virulence factor expression depending on the type of antibiotic applied. This strain was found to be protease-negative and exposing its biofilms to antibiotic did not induce protease production or down regulate protease repressors under the specified testing conditions. 1/16 MIC of roxithromycin significantly reduced the production of coagulase when compared to control. This could imply that the use of roxithromycin in treating staphylococcal infections will decrease its virulence. On the other hand, the use of cefalexin at different sub-MICs resulted in variable effects on biofilm formation. At certain range of sub-MICs, cefalexin induced biofilm formation. This indicates that there should be proper usage of cefalexin to treat staphylococcal infections, otherwise, improper usage of such antibiotics may have the unintended

consequence of promoting biofilm formation, thus, worsening the infection. There is a discrepancy in the effect of β -lactam (cefalexin) on production of TSST-1 and those obtained with Stevens *et al.* (2007). In this study, cefalexin at 1/16 MIC has reduced the production of TSST-1, while TSST-1 production increased at different sub-MICs after 12 and 24 hrs when nafcillin was used. This discrepancy could be attributed to differences between the two drugs (nafcillin is a penicillin derivative, while cefalexin is a cephalosporin). Also, in Stevens *et al.* work (2007), the bacteria were in batch culture and they were exposed to the antibiotics at mid-exponential phase, which is known to be particularly sensitive to β -lactam activity. By contrast, in this study, the bacteria were grown in biofilm and were exposed to the antibiotic from the start of biofilm formation.

Chapter 6

Purification of Toxic Shock Syndrome Toxin 1 from eluate supernatants challenged with sub-MIC of selected antibiotics and an assay of its biological activity using a mouse splenocyte model.

6.1 Introduction

The *tst* gene encoding TSST-1 is located in a mobile genetic material in the staphylococcal chromosome called the pathogenicity island (Lindsay *et al.*, 1998). As discussed earlier, *tst* gene expresses TSST-1 precursor (a 234 aminoacid protein), which has a 40 aminoacid N-terminal signal peptide (Blomster-Hautamaa *et al.*, 1986). The secretion of staphylococcal exoproteins including TSST-1 is not fully elucidated although secretory protein export in *Escherichia coli* and *Bacillus subtilis* is well described. Therefore, since most the components of exoprotein transport pathways of these organisms are conserved in *S. aureus*, knowledge from *E. coli* and *B. subtilis* was used to fill the current knowledge gaps in *S. aureus* (Sibbald *et al.*, 2006). It is believed that TSST-1 is transported via the general secretory (Sec) pathway, since it contains a signal peptide (Alksne *et al.*, 2000). Upon emergence of the nascent TSST-1 precursor from the ribosome, the signal peptide is recognized by chaperones or targeting factors in order to target the preprotein to the cytoplasmic membrane (Sibbald *et al.*, 2006). At the membrane, the precursor will be directed to the translocation machinery. The translocation machinery of *S. aureus* is composed of several (Sec) proteins. The precursor will undergo several interactions with specific Sec proteins modulated by ATP binding

and release. These interactions will lead to conformational changes that will promote the insertion of the precursor into the membrane embedded translocation channel and its subsequent release to the extracytoplasmic compartment. During or shortly after translocation, the signal peptide is cleaved by a signal peptidase leaving the protein in the unfolded state. By the aid of a folding catalyst (in case of *S. aureus* it is believed to be the lipoprotein PrsA and the membrane protein DsbA), the protein is folded rapidly into its native and protease resistant conformation. The mature protein is then secreted from the cell wall as it has no cell wall retention signal (Sibbald *et al.*, 2006).

Since the synthesis and secretion of TSST-1 involve numerous complicated steps, it is assumed that the presence of sub-MIC antibiotics may interfere with one or more of these steps resulting in attenuated TSST-1. Therefore, the first objective of this chapter is to purify TSST-1 from eluate supernatants of control biofilms and from biofilms exposed to sub-MIC antibiotics, and to compare the biological activity of the purified toxins from treated biofilms with that of control. The second objective is to test the proliferative activity of eluate supernatants (the control and treated eluates) as such and to compare the results with those obtained from purified TSST-1.

6.2 TSST-1 purification steps

The method used by Reiser *et al.* (1983) was used with some modifications in this study to purify TSST-1 from eluate supernatants taken from control biofilm and biofilms exposed to sub-MIC of antibiotics. This method has been used by others or modified to purify TSST-1 and other toxins (Reiser *et al.*, 1984; Micusan *et al.*, 1986, Su and Wong, 1995; Munson *et al.*, 1998).

S. aureus NCTC 11962 biofilms were established on Sorbarod units as described in Chapter 2. After inoculating the Sorbarods with mid-exponential phase bacterial cells,

the filters were perfused with BHI media in the case of control biofilm or BHI with sub-MIC of antibiotic. In each case three Sorbarod units were used. Once steady state was achieved (after seven hours), eluate was collected and stored at -20°C. The collection of eluate continued until 48hrs. In order to prepare cell free eluate, the frozen eluates were thawed and centrifuged at 13000 rpm at 4°C using cooled continuous centrifuge (Contifuge 17RS Heraeus Sepatech, Osterode, Germany). Eluates were pumped to the centrifuge using peristaltic pump (Autoclude, model V, England) set at 15% feeding rate. Centrifugation was repeated twice. Eluate supernatant (~3L) was collected to be used in the next step.

All buffer systems used during purification steps were prepared from distilled then deionised water (Nanopure Diamond, Barnstead, USA). The preparation of buffers was described in Chapter 2. All buffers were filtered through a 0.45µm membrane filter.

6.2.1 Batch adsorption using Amberlite CG50 (Sigma)

Amberlite CG50 is a weakly acidic cation exchanger which was used to adsorb proteins from the supernatants. Since it is a weak cation, high salt concentration may interfere with its function. Therefore, eluate supernatants should be diluted before applying them on the cation exchanger. In order to determine the suitable dilution factor, a mini scale trial was performed. In this trial, eluate supernatants were diluted in different proportions in water (1:1, 1:2, 1:3, 1:4, 1:5) and their pHs were adjusted to 5.6 by HCl. A quantity of Amberlite CG50 proportionate to the volume of supernatant to be used was prepared as described in Chapter 2. The cation exchanger was added to the diluted supernatants and mixed by magnetic stirrer for 30 minutes. After the resin settled down, the supernatant was removed and Amberlite was packed in a 5mL syringe. Several syringes were prepared, one for each dilution. The resin was washed with water and the

adsorbed proteins were eluted with 0.5M sodium phosphate buffer with 0.5M NaCl pH 6.2 by slowly dripping a volume of buffer proportionate to the amount of Amberlite. The eluted buffer was collected. Proteins in the eluted buffer and the remaining solutions (solutions from which protein was adsorbed) were precipitated by trichloroacetic acid, washed, dried and reconstituted with lysis buffer as described in Chapter 2. 2D-PAGE analysis was performed to portions taken from the eluted buffer (Figure 6.1A) and to portions taken from the remaining solutions (Figure 6.1B). The dilution that resulted in complete disappearance of TSST-1 from the remaining solution and produced highest protein spot in the eluted buffer was selected. The best dilution factor was found to be four.

The collected eluate supernatants (3L) were diluted four times in water and the pH was adjusted to 5.6 using 5M HCl. Amberlite resin was prepared as was described in Chapter 2. The swollen resin was added to the diluted eluate supernatant and stirred using (IKA RW16 basic stirrer, IKA labortechnik, Germany) for 2.5 hours at speed 5. The resin was left to settle down for 30 minutes and the supernatant was discarded. The resin with adsorbed protein was transferred to an XK 26/40 (GE Healthcare) column and washed with water (500mL) at flow rate 120mL hr^{-1} . The adsorbed proteins were eluted from the column by 2L of 0.5M sodium phosphate buffer with 0.5M NaCl pH 6.2 at a flow rate of 120mL min^{-1} . The flow rate was maintained by a two-piston pump (P-500, Pharmacia, Sweden) connected to the solvent reservoir. The eluted protein was collected and filtered under vacuum through a $10\mu\text{m}$ membrane filter (47mm polycarbonate membrane filter, Nuclepore Corporation, USA) to be ready for the next step of processing.

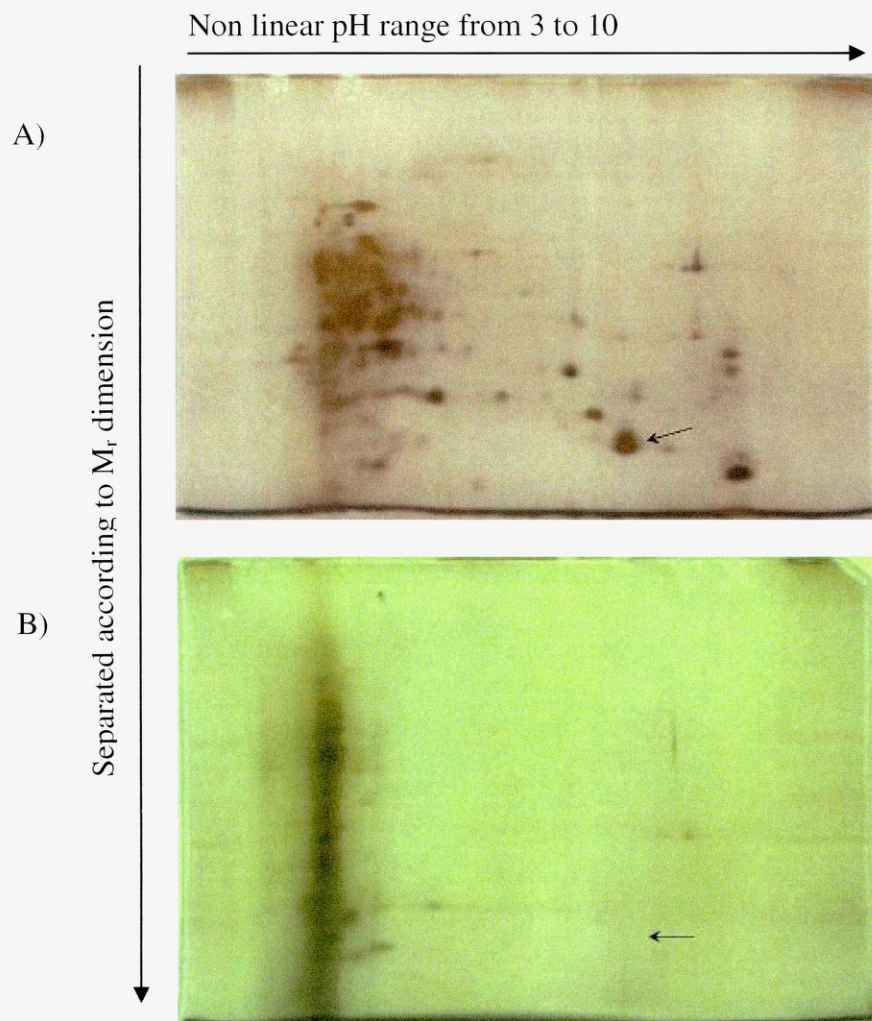


Figure 6.1: 2D-PAGE gels for the mini trial where eluate was diluted four times. (A): proteins eluted from Amberlite CG50 after being adsorbed on the resin. The arrow shows TSST-1 spot and (B): proteins in the solution remaining after batch adsorption where the arrow shows that TSST-1 disappeared (adsorbed onto the resin). Protein loading 25 μ g.

6.2.2 Concentrating the protein solution

Several methods can be used to concentrate protein solutions, such as precipitation (salting out), dialysis, gel filtration and ultrafiltration. In this study, ultrafiltration through a 10kDa molecular weight cut-off membrane was used. In ultrafiltration, a solution containing proteins and molecules with different molecular weights and sizes is forced through a membrane with a certain pore cut-off. Small molecules (such as water and low molecular weight solutes) can pass through the membrane with the ultrafiltrate, while

large molecules are retained in the retentate and concentrated relative to the starting solution (Scopes, 2002).

The eluted protein solution (2L) was forced through a regenerated cellulose ultrafiltration membrane device (Pellicon XL device, Millipore, USA) using a peristaltic pump (Masterflex, Cole Parmer Instrument Company, USA). The solution was circulated through the membrane until the volume was reduced to 50mL. In order to replace the buffer system of the protein solution and simultaneously remove excess low molecular weight solutes, dialysis was performed (Scopes, 2002). The concentrated protein solution was transferred to dialysis tubing (12 to 14kDa) molecular weight cut-off (Visking dialysis tubing, Medicell International Ltd, UK). Dialysis tubing was previously washed and prepared according to manufacturer's instructions. Dialysis was performed by four changes of 5mM phosphate buffer pH 5.6 for 24hr at 4°C.

6.2.3 Ion exchange chromatography using CM-Sepharose CL 6B

A chromatography column (XK 26/40; GE Healthcare) containing CM-Sepharose CL 6B ion exchanger was prepared, washed and equilibrated as was discussed in Chapter 2. The entire dialysed protein sample solution (~50mL) was introduced to the column. The outlet of the column was connected to a monitor set at 280nm (Single path monitor UV-1 optical unit and Single path monitor UV-1 Control unit, Pharmacia, Sweden). The effluent from the monitor was collected in 20mL fractions by a fraction collector (LKB Bromma, model 2070, Ultrorac II, Sweden). The resin was washed by pumping 500mL water at flow rate 120mL hr⁻¹. The toxin was eluted from the column by applying three different buffers. The buffers were delivered to the column in succession using two pumps (P-500) controlled via Gradient programmer (GP-250, Pharmacia, Sweden). The buffers percolated into the column at a flow rate 60mL hr⁻¹ according to the following

order: 500mL of 5mM sodium phosphate buffer pH 6.0, 2250mL of 0.03M sodium phosphate buffer pH 6.0 and 1100mL of 0.045M sodium phosphate buffer pH 6.4.

Proteins eluting in the effluent were estimated by measuring the absorbance of the collected fractions at 280nm (Figure 6.2) using UV/Vis spectrophotometer (Genesys 10UV, Thermo Spectronic, New York, USA).

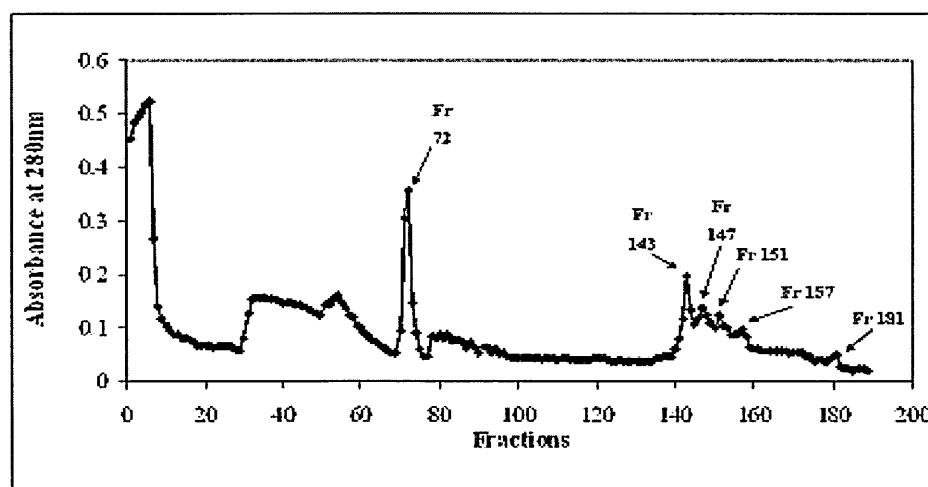


Figure 6.2: An example of ion exchange chromatogram on CM-Sepharose CL 6B for control supernatants. Fractions (20mL) were collected and absorbance at 280nm was measured. Arrows indicate fraction (Fr) number at the apex of the peak.

In order to determine the fractions containing TSST-1, SDS-PAGE analysis was performed on aliquots taken from the fractions corresponding to peaks. Figure (6.3) shows SDS-PAGE gel of the fractions forming the peaks. Fraction 147 was found to contain TSST-1, therefore, fractions forming this peak (fractions 146 to 149) were collected, mixed and dialysed against 40% w/v polyethylene glycol (PEG) 20000 at 4°C for 24hr in order to concentrate TSST-1.

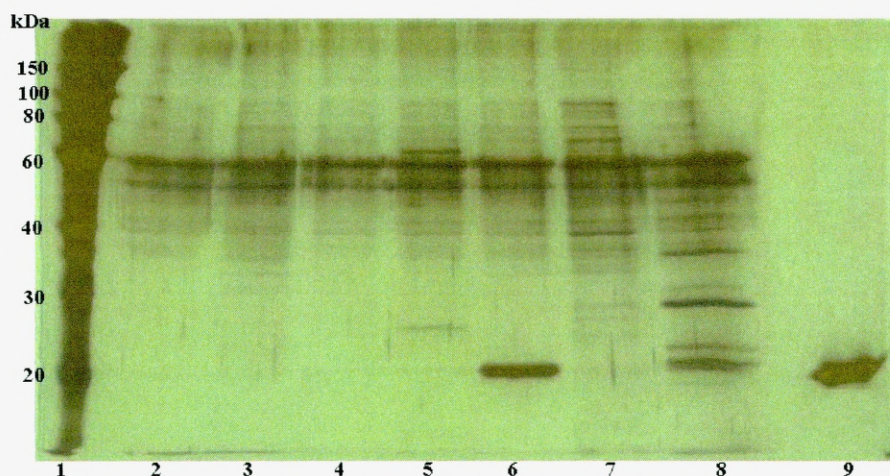


Figure 6.3: SDS-PAGE analysis of the fractions corresponding to the eluted peaks from CM-Sepharose CL 6B (Figure 6.2). Lane 1, SDS-PAGE protein marker; lane 2, fraction 181; lane 3, fraction 157; lane 4, fraction 153; lane 5, fraction 151; lane 6, fraction 147; lane 7, fraction 143; lane 8, fraction 72; lane 9, TSST-1 standard. Volume applied: maximum well capacity (23 μ L).

At this stage, TSST-1 was not detected in fractions eluted from supernatants exposed to roxithromycin. This could be related to reduced column efficiency and loss of resolution since the exposed roxithromycin supernatant was purified last.

6.2.4 Gel Filtration using Sephacryl S 200HR

The concentrated toxin (~3mL) from the previous step was filtered through a 0.45 μ m nylon membrane filter. The sample was injected into a column of Sephacryl S 200HR (XK 26/100) through sample application valve (V-7, Pharmacia, Sweden). The column was previously packed, washed and equilibrated with 0.05M phosphate buffer pH6.8 containing 1M NaCl (described in Chapter 2). The toxin was eluted from the column by applying 600mL of 0.05M sodium phosphate buffer pH 6.8 containing 1M NaCl at a flow rate of 60mL hr⁻¹ and the eluted effluent was collected in 5mL fractions. Aliquots from the fractions were tested spectrophotometrically at 280nm (Genesys 10UV, Thermo Spectronic, New York, USA). Figure 6.4 shows fractions eluted from gel filtration column (Sephacryl S 200HR). Fractions from the eluted peaks were analysed by

SDS-PAGE to identify those containing TSST-1 (Figure 6.5). Fractions containing TSST-1 were combined together and dialysed at 4°C against 5mM phosphate buffer pH 6.8 using Visking dialysis tubing (Medicell International Ltd, UK) (12 to 14kDa) molecular weight cut-off. The purified toxin was distributed in small tubes and stored at -20°C for subsequent use.

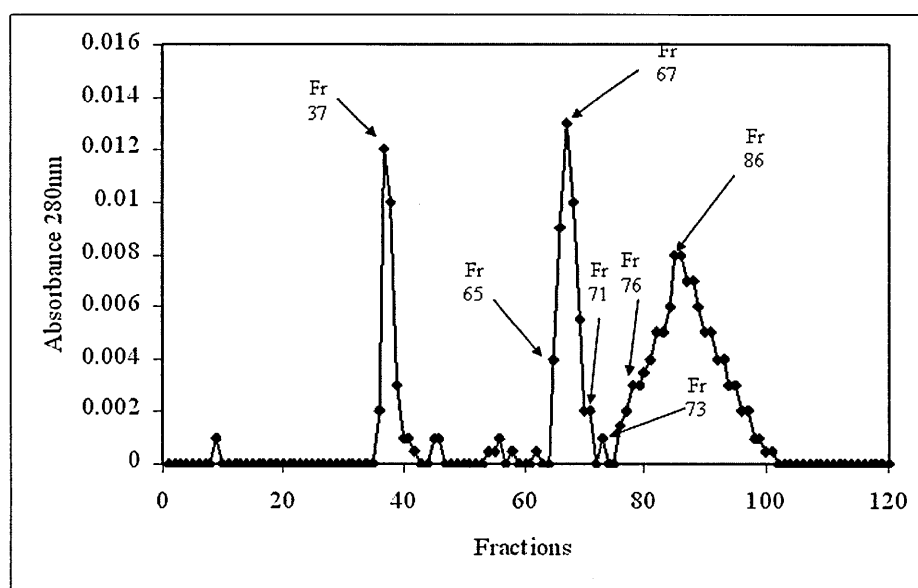


Figure 6.4: An example of gel filtration chromatogram on Sephacryl S 200HR (Sigma) for control supernatants, (5mL) fractions were collected and absorbance at 280nm was measured. Arrows indicate fraction (Fr) number.

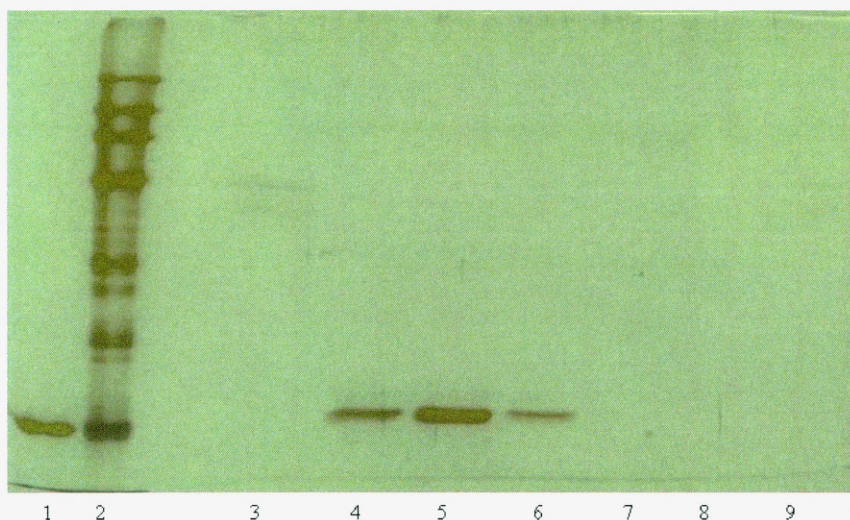


Figure 6.5: SDS-PAGE analysis of fractions eluted from gel filtration column (Sephacryl S200 HR; Figure 6.4). Lane 1, TSST-1 standard (sigma); lane 2, SDS-PAGE marker; lane 3, fraction 37; lane 4, fraction 65; lane 5, fraction 67; lane 6, fraction 71; lane 7, fraction 73; lane 8, fraction 76; lane 9, fraction 86. Volume applied: the maximum well capacity (23 μ L well⁻¹)

6.3 Testing the biological activity of TSST-1 *in vitro*

TSST-1 biological activity can be evaluated *in vivo* and *in vitro* based on its activity as a superantigen. *In vivo* studies may include studying the lethal effect of TSST-1 in rabbits (Reeves *et al.*, 1986; Kum *et al.*, 1993) or mice (Stiles *et al.*, 1995), changes in blood chemistries of infected rabbits (Bonventre *et al.*, 1993), cytokine release (interleukins, tumour necrosis factors, interferons) (Stiles *et al.*, 1995). *In vitro* studies of TSST-1 involve studying the production of cytokines by human lymphocytes (Micusan *et al.*, 1986; Parsonnet and Gillis, 1988; Akatsuka *et al.*, 1994; Krakauer and Stiles, 1999; Imanishi *et al.*, 2003), rabbit lymphocytes (Ikejima *et al.*, 1988), murine lymphocytes (Micusan *et al.*, 1986) and bovine lymphocytes (Yokomizo *et al.*, 1995) in addition to studying the proliferative effect of TSST-1 on different types of lymphocytes whether from human or animal origin (Calvano *et al.*, 1984; Yokomizo *et al.*, 1995; Krakauer and Stiles, 1999; Gampfer *et al.*, 2002).

As discussed in Chapter 1, TSST-1 as a superantigen, has a mitogenic effect on T-lymphocytes. It binds to major histocompatibility complex II (MHC II) of the antigen presenting cell (APC) outside the conventional binding site and the complex binds to specific V β element of the T-lymphocyte outside the binding groove. This binding will stimulate T-lymphocytes to proliferate and triggers the release of cytokines from APCs and lymphocytes (Dinges *et al.*, 2000; Lee and Bohach, 2004). Therefore, the presence of APCs is important for the superantigenic activity of TSST-1. See *et al.* (1992), have shown that TSST-1 was not able to produce interleukin-1 or tumor necrosis factor when lymphocytes or monocytes were exposed to it separately. However, a 1:1 mixture of these cells resulted in significant production of these cytokines (See *et al.*, 1992). Similarly, presence of macrophages has been proven to be necessary for the proliferation of lymphocytes in mouse and human systems (Poindexter and Schlievert, 1985). These properties (proliferative effect of TSST-1 on lymphocytes and provoking the release of cytokines) were used to measure the activity of TSST-1 *in vivo* and *in vitro* under different conditions (Calvano *et al.*, 1984; Blanco *et al.*, 1990; Bonventre *et al.*, 1993; Kum *et al.*, 1993; Stiles *et al.*, 1995; Krakauer and Stiles, 1999). On the other hand, although TSST-1 has a lethal effect on animals and humans, it was shown not to possess cytotoxic activity on eukaryotic cells (Deresiewicz *et al.*, 1994).

In this project, the proliferative effect of TSST-1 purified from eluate supernatants and the proliferative effect of eluate supernatants exposed to different treatments and control will be studied on freshly prepared mouse splenocytes.

6.3.1 Mouse splenectomy

Adult female Swiss mice (six to nine weeks old) were obtained from a colony maintained by Ninewells Hospital, Dundee, after being freshly sacrificed by cervical

dislocation. The mouse was then laid on its right side on a clean chopping board, which was disinfected with 70% alcohol. The mouse skin was wetted with 70% alcohol. The dissection tools were sterilized by autoclaving. The skin was cut midway on the mouse's left side. The peritoneum was carefully lifted with small forceps and cut to expose the spleen. The spleen was excised from the abdomen and mesenteries by a scalpel and then placed in a sterile Petri dish (5cm, Sterilin) containing about 5mL sterile Dulbecco's phosphate buffered saline pH 7.2 (D-PBS) (NaCl (8.0g), KCl (0.2g), KH_2PO_4 (0.2g), Na_2HPO_4 (1.15g) and water up to 1L, sterilized by autoclaving).

6.3.2 Preparing single cell suspension of splenocytes

For each experiment, two spleens were excised. Each was treated separately to extract splenocytes, but in the final two washes, splenocytes were combined.

Two sterile 5mL syringe needles were bent at right angles using the protective cap of the needle. One needle was used to hold the spleen to the dish, the other one was used to make an incision in the spleen capsule and to express cells from the spleen. A sterile Pasteur pipette was used to break up the cells clumps by repeated aspiration and blowing of cell suspension. Then the whole dish contents were passed through sterile 70 μm mesh (Cell strainer BD Falcon, BD Biosciences, USA). The filtrate was collected in sterile 50mL tube. The mesh containing the retained clumps and spleen remains was placed in petri dish containing fresh 5mL PBS. The rubber end of a sterile 3mL syringe plunger was used to break up clumps and to gently squeeze cells from the spleen against the cell strainer. This was performed until only white connective tissue was left from the spleen fragments.

The contents of the dish were passed through the 70 μm mesh and added to the previous splenocyte suspension. The dish was washed twice with 2mL of fresh D-PBS

and the washings were added to the suspension. The suspension was transferred to a 15mL conical centrifuge tube and the cells were pelleted by centrifugation at 4000 rpm for 2min (IEC Centra-4B Centrifuge, International Equipment Company, USA). The supernatant was discarded. The pellet was loosened by flicking the ends of the tube and 1mL of sterile red blood cells (RBC) lysis buffer was added to it (RBC lysis buffer: 0.83% ammonium chloride in 0.01 M Tris-HCl buffer, pH 7.2, filter sterilized; Czajgucki *et al.*, 2007) and then incubated at room temperature for 5 min. The activity of lysis buffer was terminated by adding chilled D-PBS up to 15mL. The suspension was centrifuged at 4000 rpm for 2min (IEC Centra-4B Centrifuge, International Equipment Company, USA) and the supernatant was discarded. In order to lyse the residual red blood cells, the procedure was repeated twice. Later the cells of the two spleens were combined together and washed twice with sterile tissue culture medium. Tissue culture medium was composed of sterile RPMI 1640 with L-glutamine and sodium bicarbonate (Sigma) containing sterile 5% Foetal bovine serum (Biowhittaker Lonza-Belgium) and sterile RPMI 1640 Vitamins solution (100x) (Sigma), 100 μ g mL⁻¹ streptomycin as sulphate (Sigma) (stock solution prepared and filter sterilized), 100 U mL⁻¹ penicillin G as sodium salt (Sigma) (stock solution prepared and filter sterilised).

Washing the cells was done by adding 10 mL of tissue culture medium to the cells, swirling the tube gently, pelleting the cells by centrifuge and discarding the supernatant. In the second wash after adding the 10mL medium, the suspension was passed through glass wool packed into a 3mL syringe previously sterilised by autoclaving, to remove the cell debris of the lysed RBCs (cell debris will attach to glass wool while splenocytes do not) (Czajgucki *et al.*, 2007). The suspension was centrifuged and the supernatant discarded. After washing, the cells were transferred to a 50mL sterile tube and suspended in 30mL tissue culture medium.

6.3.3 Adjustment of splenocytes density (cells mL⁻¹)

Trypan Blue exclusion assay was used to adjust cell density to 5×10^6 cell mL⁻¹ (Blanco *et al.*, 1990). The cells were mixed gently in the tube and 50 μ L aliquot was mixed with 50 μ L Trypan Blue (0.4% w/v) (Sigma). The Trypan blue-cell suspension mixture was applied to a haemocytometer (Neubauer improved) where the mixture spread by capillary action under the glass cover and filled the whole chamber. Viable cells exclude Trypan blue dye, whereas dead cells take up the dye and attain dark blue colour (Micusan *et al.*, 1986; Lyu and Park, 2006). Viable cells were counted in each of the four primary squares of haemocytometer chamber using 100x magnification, and the sum of viable cells in the four primary squares was calculated. Cell density (cells mL⁻¹) in the suspension was calculated using the following formula:

$$D = N \times 5000 \quad (\text{equation 6.1})$$

Where D: Cell density; N: total number of cells in the 4 primary squares and 5000 is the result of calculating the volume of suspension retained in 4 primary squares (each with 1 mm² area x 0.1 mm depth) and the dilution of the suspension in 1:1 ratio with Trypan blue. According to the resulting cell density, the suspension was diluted by adding fresh tissue culture medium to obtain cell concentration of 5×10^6 cells mL⁻¹.

6.3.4 Proliferation assay using MTS/PMS method

MTS is a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, salt. PMS (phenazine methosulfate) is an electron coupling reagent (Cory *et al.*, 1991). The MTS/PMS assay is a colourimetric method for determining the number of viable cells in proliferation assays. It is widely used in research for *in vitro* cell growth assays in human, animal and algal cells (Capasso *et al.*, 2003; Ganguly *et al.*, 2006; Pi *et al.*, 2006).

Viable cells are metabolically active and produce reducing agents such as NADH or NADPH. These compounds pass their electrons to an electron coupling reagent (PMS), which reduces the tetrazolium compound MTS into a soluble intensely coloured formazan product, whose optical density can be determined in the range 450 to 540nm, optimum wavelength 490nm (Cory *et al.*, 1991; Goodwin *et al.*, 1995; Chen *et al.*, 2002; Anon, 2005). Dead cells cannot reduce tetrazolium products, therefore formazan compound production is proportional to the number of viable cells in culture.

When MTS/PMS is compared to other microculture tetrazolium salts assays like XTT (2,3 - bis (2-methoxy- 4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); MTS/PMS and XTT/PMS were shown to have the advantage of forming soluble formazan product which eliminates possible errors in the steps of solubilisation required in MTT assay (Cory *et al.*, 1991). On the other hand, XTT/PMS mixture shows instability in some solutions, which results in poor assay precision. This instability was not encountered in MTS/PMS assay (Goodwin *et al.*, 1995).

Being a non-radioactive assay, MTS/PMS has the advantage over tritiated thymidine incorporation in that it is safe and does not require scintillation cocktails or disposal of radioactive waste. In a study by Zolnai *et al.* (1998), to determine mitogen-induced lymphocyte proliferation in cell culture, the results of MTS/PMS showed good correlation with tritiated thymidine incorporation assay.

6.3.4.1 Purified TSST-1 proliferation assay

The concentration of purified TSST-1 from biofilm eluate supernatants of cefalexin and ciprofloxacin treatments and a control was determined by the Bradford assay method (described in Chapter 2). The concentrations were 4.6, 4.3 and 4.7 $\mu\text{g mL}^{-1}$

respectively. Purified TSST-1 from the treatments and control was diluted into tissue culture medium to a concentration of 2000ng mL^{-1} . Ten fold serial dilutions were done to each purified toxin in tissue culture medium to give 200, 20, 2, 0.2 ng mL^{-1} in addition to the stock which is 2000ng mL^{-1} . Aliquots of $100\mu\text{L}$ were taken from each toxin concentration of each treatment and distributed in sterile 96-well flat-bottom tissue culture plate (Fisher Scientific, England). Each concentration was applied in triplicate.

Phytohemagglutinin PHA-P (Lectin from *Phaseolus vulgaris* (red kidney beans) containing buffer salts and NaCl) (Sigma) was used as mitogen positive control. 5mg of the lyophilised PHA-P powder was dissolved in 5mL sterile water. A stock solution of $80\mu\text{g mL}^{-1}$ was prepared by dilution in tissue culture medium. Aliquots of $100\mu\text{L}$ from the stock were distributed in three wells in a 96-well flat-bottom tissue culture plate. Double serial dilution was done by adding $100\mu\text{L}$ aliquots from the stock to other wells containing $100\mu\text{L}$ fresh medium in triplicates. The solution was mixed and $100\mu\text{L}$ transferred to other wells containing fresh medium. The process was repeated until a concentration range from 80 to $10\mu\text{g mL}^{-1}$ was obtained where the last $100\mu\text{L}$ taken from the last wells after mixing were discarded. In order to make negative control, $100\mu\text{L}$ of fresh medium were added to new wells. Aliquots ($100\mu\text{L}$) of 5×10^6 splenocytes were added to all the wells so that, the final concentration of TSST-1 in the wells ranged from 1000 to 0.1ng mL^{-1} , whereas the final concentration of PHA-P in the wells ranged from 40 to $5\mu\text{g mL}^{-1}$. Splenocytes ($100\mu\text{L}$) were also added to the negative control wells. Accordingly, the concentration of cells present in all the wells was 5×10^5 cells well⁻¹. The plates were covered and incubated at 37°C , 5% CO_2 incubator (Heraeus Instruments, Model B5060 EK/ CO_2 , Germany) for 48 hours. After the incubation period, $40\mu\text{L}$ of MTS/PMS dye mixture (Cell Titer 96 Aqueous Non Radioactive Cell proliferation assay, Promega, USA) (prepared by adding 1mL of PMS solution into 20mL MTS solution) was

added to all wells and incubated for 4 hours at 37°C, 5% CO₂ according to manufacturer's recommendation.

In order to measure the optical density of formazan product converted by viable splenocytes from tetrazolium salts, the plates were mixed gently for 30 seconds and optical density (OD) was measured at 450nm using ELISA reader (Rosys Anthos ht III- Anthos Labtec Instruments, Salzburg). OD of the negative control was subtracted from those of samples and PHA-P. The concentration of PHA-P which produced the highest response (OD value) was selected and its average value was considered 100% response. OD results of the samples were calculated as percentage response from PHA-P. The results were expressed as mean \pm standard deviation (SD) and plotted versus concentration of the mitogen (toxin or PHA).

6.3.4.2 Statistical analysis

Statistical difference among the results obtained by the treatments and control were examined by one way analysis of variance (ANOVA), followed by Tukey multiple intergroup comparison. P values < 0.05 were considered significant. Normal distribution of the results and equal variance were checked using Bartlett's test ($p > 0.05$) and Anderson-Darling test ($p > 0.05$). All calculations were carried out using Minitab 15 software.

6.3.4.3 Results and discussion

Figure 6.6 shows the proliferative effect of the positive control mitogen, phytohemagglutinin (PHA-P). After 48 hr incubation with mouse splenocytes, the 10 μ g mL⁻¹ concentration produced the highest response. This indicates the presence of an optimal concentration for the mitogen to produce the highest response. The response (OD

450nm) of splenocytes at this concentration was considered as 100% response. This behaviour is consistent with that obtained with concanavalin A (a positive mitogen) when used in splenocyte proliferation assay (Lyu and Park, 2006), where an optimal dose produced the highest proliferation above which a reduction in proliferation happened.

Figure 6.7 shows the proliferative effect of TSST-1 purified from eluate supernatants exposed to sub-MIC cefalexin and ciprofloxacin and control. In the three cases, the proliferative effect of the toxin increases in a dose dependent manner until a peak (optimum concentration) is reached, after which the response is decreased. This proliferative effect of TSST-1 is supported by the work of others as follows: the increase in splenocyte proliferation as a result of increase in the toxin concentration was obtained by Bonventre *et al.* (1993), who studied the toxicity of recombinant TSST-1 and mutant toxins on mouse splenocytes at concentration range from 0.1 to 100ng mL⁻¹, as the toxin concentration increases, tritiated thymidine incorporation increased. In another study, TSST-1 purified from periplasmic space of *E. coli* after cloning the TSST-1 gene in it, exhibited an increase in mitogenic activity on mouse splenocytes at concentrations ranging from 1 to 500ng mL⁻¹ (Blanco *et al.*, 1990). Similarly, the mitogenic effect of TSST-1 on peripheral blood mononuclear cells (PBMC) has been shown to be dose dependent (Kum *et al.*, 1993).

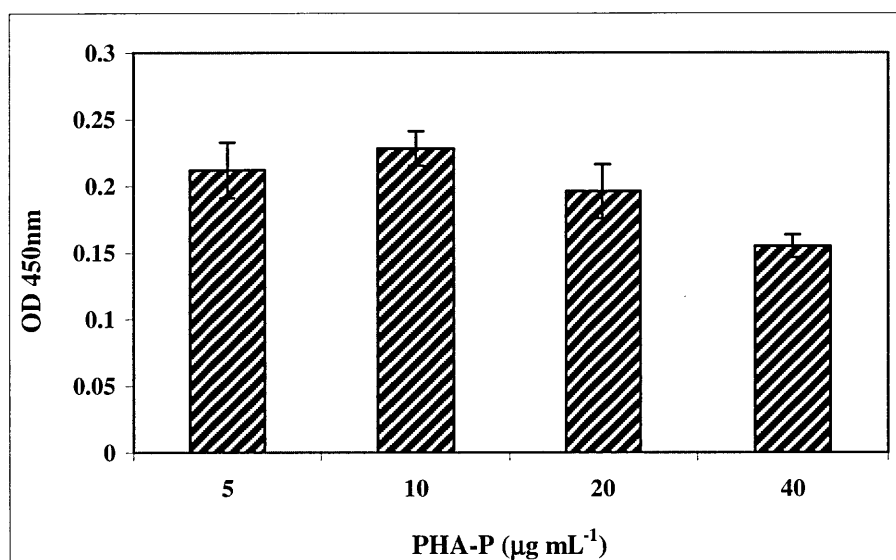


Figure 6.6: Proliferative effect of the mitogen PHA-P on mouse splenocyte. MTS/PMS assay was performed and optical density (OD at 450nm) was plotted against different concentrations of PHA-P. Error bars indicate \pm standard deviations from the mean (n=3).

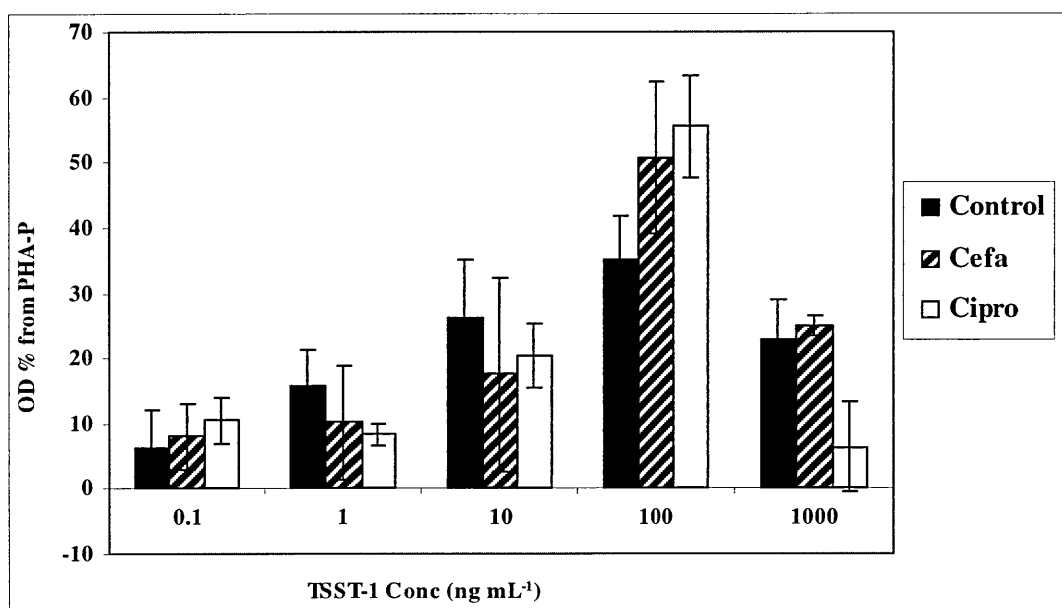


Figure 6.7: Proliferative effect of purified TSST-1 from biofilm eluates of control, cefalexin (cefa) and ciprofloxacin (cipro) on mouse splenocyte. Optical density (OD) at 450nm was measured and OD of negative control was subtracted. OD of the samples was calculated as percentage from OD of PHA-P. Error bars indicate \pm standard deviations from the mean (n=3).

At the concentration range from 0.1 to 100ng mL⁻¹, TSST-1 purified from control biofilm eluates or from treated biofilm eluates was found to possess comparable proliferative effect at each concentration level (Table 6.1). There is no significant difference among the two treatments and control at 95% confidence level (Table 6.1). At 1000ng mL⁻¹, the reduction in response was higher for TSST-1 purified from supernatants exposed to ciprofloxacin.

Table 6.1: Tukey's multiple group analysis (95% confidence) of the proliferative effect of TSST-1 purified from eluates of control, cefalexin treated and ciprofloxacin treated biofilms on mouse splenocytes. Statistical analysis was performed between treatments. Treatments with similar effect were given same letter. Treatment which is significantly different from the others was given different letter.

TSST-1 Conc. ng mL ⁻¹	Control	Cefalexin	Ciprofloxacin
0.1	A	A	A
1	A	A	A
10	A	A	A
100	A	A	A
1000	A	A	B

6.3.4.4 Eluate supernatants proliferation assay

Eluate supernatants of ciprofloxacin, cefalexin and roxithromycin treatments and control were diluted in ten fold serial dilutions with tissue culture medium to obtain final dilution in the range of 5×10^{-2} to 5×10^{-5} . A dilution of 1/2 was excluded from the study due to high interference of BHI medium present in eluate supernatants on the MTS/PMS OD values. Aliquots (90µL) of tissue culture medium were placed in the wells of 96-well

flat-bottom tissue culture plate. Eluate supernatants (10 μ L) of each treatment and control were placed in the wells in triplicates. The contents were mixed and 10 μ L were transferred to the next wells containing 90 μ L tissue culture medium. The process was repeated until 1×10^{-4} dilution of eluate supernatant in tissue culture medium was obtained. Splenocyte suspension (90 μ L of 5×10^6 cells mL $^{-1}$) was added to all wells. A negative control was prepared as was described earlier. PHA-P was prepared at a concentration which gave the highest response in the previous experiment (10 μ g mL $^{-1}$). The plates were covered and incubated at 37°C, 5% CO $_2$. After 48hrs, 36 μ L of MTS/PMS dye mixture was added to all wells and incubated for 4 hours at 37°C, 5% CO $_2$. The plates were mixed gently for 30 seconds and optical density (OD) was measured at 450nm using an ELISA reader. The OD of the negative control was subtracted from those of samples and PHA-P. OD results of the samples were calculated as percentage response from PHA-P. The results were expressed as mean \pm standard deviation (SD) and plotted versus the dilution.

6.3.4.5 Results and discussion

Figure 6.8 shows the proliferation of mouse splenocytes when incubated with different dilutions of eluate supernatants of control biofilms and biofilms exposed to sub-MIC antibiotics. The highest response was obtained at 5×10^{-3} dilution and as the dilution increased the response decreased indicating that the response is dose dependent. However at the lowest dilution (5×10^{-2}), which is the highest concentration of mitogens, the response was the lowest indicating the presence of an optimal mitogenic effect after which the response declines. As mentioned earlier, 1/2 dilution was excluded from the study due to high interference from BHI on the OD of MTS/PMS dye. These results are consistent with those obtained with purified TSST-1, as after an optimal concentration, reduction to proliferation appeared.

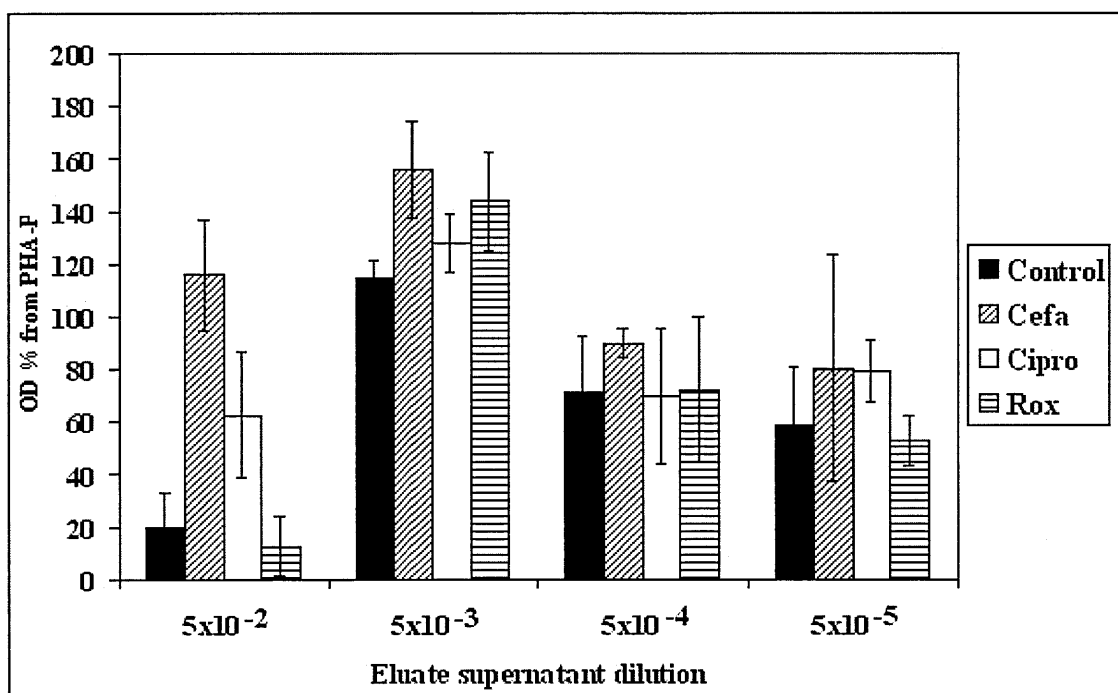


Figure 6.8: Proliferative effect of eluate supernatants exposed to cefalexin (Cefa), ciprofloxacin (Cipro) and roxithromycin (Rox) on mouse splenocytes. The activity was calculated as a percentage from PHA-P activity, assuming the activity of PHA-P to be 100%. Error bars indicate \pm standard deviations from the mean (n=3).

At high dilutions (5×10^{-4} and 5×10^{-5}) there was no significant difference in the proliferative effect among the treated and control eluates (Table 6.2). At 5×10^{-3} dilution, the effect of cefalexin treated eluate was significantly different from control eluates ($p < 0.05$), although it was not significantly different from ciprofloxacin or roxithromycin treated eluates. At 5×10^{-2} dilution, the proliferative effect of cefalexin treated eluates was significantly different from the control and other treatments. The concentration of TSST-1 in eluate supernatants was estimated earlier (Chapter 5, section 5.4.2), accordingly, the concentration of TSST-1 in the diluted supernatants is calculated (Table 6.3).

From tables 6.2 and 6.3, it is noticeable that cefalexin at 5×10^{-3} and 5×10^{-2} dilution has more significant mitogenic effect than the control although TSST-1 concentration in it is almost quarter that in the control supernatant. The same can be said about ciprofloxacin where the content of TSST-1 is half that in control, but still its mitogenic effect is

comparable to that of control. This effect could imply that either TSST-1 in cefalexin treated supernatants is more potent than that in control supernatant or that another factor in the eluate is potentiating the mitogenic effect of cefalexin treated supernatant and that its effect is more pronounced at lower dilutions (5×10^{-3} and 5×10^{-2}). The first explanation can be ruled out because it has been shown in Table 6.1 that TSST-1 biological activity is the same whether it was purified from control or treated eluates. Thus, the second explanation which is the presence of other factors that contribute to the mitogenic activity is more valid.

Although it is inaccurate to compare the values of responses obtained from eluate supernatants with those obtained from purified toxins due to the fact that the two experiments were performed in different days using different splenocyte suspensions, it is obvious that by comparing the responses of purified TSST-1 of any treatment with those of corresponding eluate supernatants containing equivalent concentration of TSST-1, the response resulting from eluate supernatant is higher (higher values of OD%) (Figure 6.7 and Figure 6.8). This increase in mitogenic effect of eluate supernatants when compared to that of purified toxin can be justified by two reasons: as was mentioned earlier, *S. aureus* NCTC 11962, produces enterotoxin A. This toxin has superantigenic activity and therefore, has proliferative effect on lymphocytes (Lee and Bohach, 2004). It acts by binding to specific family of V_{β} chains that are different from those that bind to TSST-1 (Davison *et al.*, 2000). The other reason is that purified TSST-1 has passed through several steps of purification and concentration, hence, its biological activity could have been affected by these steps.

Table 6.2: Tukey's multiple group analysis (95% confidence) of the proliferative effect of eluate supernatants of control, cefalexin treated and ciprofloxacin treated biofilms on mouse splenocytes. Statistical analysis was performed between treatments. Treatments with similar effect were given same letter, treatment which is significantly different from the others was given different letter.

Eluate supernatant Dilution	Control	Cefalexin	Ciprofloxacin	Roxithromycin
5×10^{-2}	AB	D	B	AC
5×10^{-3}	BC	A	AB	AC
5×10^{-4}	A	A	A	A
5×10^{-5}	A	A	A	A

Table 6.3: Estimated concentration of TSST-1 (ng mL^{-1}) in eluate supernatants exposed to different treatments at different dilution ratios.

Eluate supernatant Dilution	Control	Cefalexin	Ciprofloxacin	Roxithromycin
5×10^{-2}	102.40	25.60	51.20	102.40
5×10^{-3}	10.24	2.56	5.12	10.24
5×10^{-4}	1.02	0.26	0.51	1.02
5×10^{-5}	0.10	0.03	0.05	0.10

6.4 Conclusion

The production and secretion of TSST-1 from *S. aureus* involves multiple steps that require the interaction and involvement of several enzymes and regulatory proteins. It was postulated therefore, that exposing *S. aureus* biofilm to sub-MIC antibiotic could have an impact on some of these steps resulting in the secretion of a protein with altered biological activity. In this study, TSST-1 was purified from eluate supernatants of biofilms exposed to sub-MIC of antibiotics and control. The biological activity of TSST-1 was tested by comparing the proliferative effect of different concentrations of the purified

toxins on mouse splenocytes. In addition, the proliferative activity of crude eluate supernatants exposed to sub-MIC was tested also by comparing the activity at different dilutions. In both cases, the proliferation of splenocytes was dose dependent up to a maximum after which the effect was reduced. There was no significant difference in the proliferative activity among purified TSST-1 from control and treated eluates at different concentrations of TSST-1 up to the optimum concentration, indicating that 1/16 MIC of cefalexin or ciprofloxacin has no significant impact on the stages involved in the synthesis of TSST-1 that would lead to altered activity. On the other hand, the proliferative activity of crude eluate supernatants exposed to cefalexin at the optimum concentration was higher than that of control. In addition, the proliferative activity of crude eluate supernatants was higher than that of purified toxins exposed to same treatment. These effects were attributed to the presence of another mitogenic agent, enterotoxin A, produced by this strain that could have been affected by the presence of cefalexin more than the presence of other antibiotics. Moreover, the possible effect of purification stages on reducing the activity of purified toxins can not be ruled out.

Chapter 7

General Discussion

The fact that staphylococci are common colonizers of human skin and mucous membranes, makes them important pathogens in causing infections once these barriers are disrupted. *Staphylococcus aureus* infections are difficult to manage, often need weeks of antimicrobial chemotherapy (Higashi and Sullam, 2006). The ability of staphylococci to form biofilms on both native tissue and abiotic surfaces inside the body comprises a challenge that faces clinicians. Biofilm structures are recognized for their high resistance to chemotherapy and the bacteria released from these biofilms cause metastatic infections that will complicate the diagnosis of the actual site of infection. If biofilm infections are not treated aggressively, the infections can recur, hence, in most cases, successful cure requires surgical removal of the biofilm.

The nutritional requirements of *S. aureus* are relatively complex when compared to the nutritional requirements of other bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*. While the later is ubiquitous in nature, the natural habitat of the staphylococci is limited to animal skin and mucosal membranes or as contaminants in food. Therefore, whilst simple sources of organic carbon, iron, magnesium and other basic ions are satisfactory to support the growth and biofilm formation of both *E. coli* and *Ps. aeruginosa* (Al-Hmoud, 2002; Masadeh, 2005) this is not the case with *S. aureus*, where several amino-acids were found to be required in order to support growth (Chapter 3). Moreover, as was exhibited in Chapter 3, even more complex media, such as brain heart infusion media, were required by the bacteria to form biofilm structures.

S. aureus is known to express numerous virulence factors that help the bacterium to invade and infect the body, to evade host defence mechanisms and to adapt to different and potentially hostile environments. These factors have made *S. aureus* among the five

most common isolates in clinical microbiology laboratories (Higashi and Sullam, 2006). Virulence factors of *S. aureus* have attracted many researchers to study the mechanism and role of these factors in infection and spread of microorganism (Hienz *et al.*, 1996; Lee *et al.*, 2002; Mack *et al.*, 2004; Shannon *et al.*, 2005; Fitzgerald *et al.*, 2006). Besides, many studies (Lebeau *et al.*, 1994; Chan *et al.*, 1998; Bischoff *et al.*, 2001; Dunman *et al.*, 2001; Lyon *et al.*, 2002; Valle *et al.*, 2003; Boisset *et al.*, 2007) have been conducted to identify regulatory pathways for these factors and the agents that have an impact on their production or regulation.

7.1 Encountering sub-MICs of antibiotics in clinical settings

Clinically, microorganisms can encounter sub-minimal concentrations of antibiotics on several occasions. Pharmacokinetic factors, such as absorption of the drug from site of application, distribution into diverse tissues, metabolism and clearance of the drug could lead to gradients in the concentration of the drug in the body tissues. In addition, pharmacological factors, such as drug-drug or drug-food interactions, certain health and clinical conditions of the patient, or direct effect of the microorganism, whether pathogenic or normal flora, especially by releasing antibiotic inactivating enzymes (Baquero, 2001), would create an environment in which bacteria as planktonic or biofilm phenotypes will face sub-inhibitory levels of antibiotics.

Understanding the clinical importance of sub-MIC of antibiotics, it was the aim of this thesis to study the effect that subinhibitory concentrations of selected antibiotics would have on the virulence factors expressed by *S. aureus* biofilms. The selected antibiotics were of three different antibacterial groups, cefalexin (a cephalosporin that inhibits cell wall synthesis), ciprofloxacin (a fluoroquinolone that inhibits bacterial DNA replication) and roxithromycin (a macrolide that inhibits protein synthesis).

7.2 Protein profile shifts may reflect alterations in bacterial virulence

Allowing *S. aureus* NCTC 11962 to grow and build a biofilm in the presence of 1/16 MIC of the antibiotics resulted in remarkable shifts in protein profiles of biofilm cells, eluate cells and in the secreted proteins (eluate supernatants) (Chapter 4). Some novel proteins appeared in SDS-PAGE and 2D-PAGE gels and others disappeared when compared to control protein profiles (Figures 4.3 to 4.17 and Tables 4.2 to 4.16). Protein shifts could be attributed to changes in virulence or strategic shifts by the organism to make changes in structural proteins and enzymes that are required for cell viability and maintenance. Changes in protein profiles of eluate supernatants were found to be accompanied by changes in some of the studied virulence factors. For example, roxithromycin at sub-MIC resulted in reduction of coagulase production when compared to control (Chapter 5, Figure 5.1). Toxic Shock Syndrome Toxin 1 production was altered when the biofilms were exposed to cefalexin and ciprofloxacin (Chapter 5, section 5.4). In another experiment (Chapter 5, section 5.3), the formation of biofilms on microtiter plates was enhanced in the presence of a range of sub-MICs of cefalexin. Taken together, these alterations in secreted virulence factors (coagulase, TSST-1 and exopolysaccharide material) will result in the appearance of different protein profiles from those secreted from control cells, in addition to the fact that variations in virulence should reflect intracellular changes in some regulatory proteins or effector agents. Such changes will be observed as different protein patterns on electrophoresis gels.

7.3 Effect of subinhibitory concentrations of antibiotics on virulence

It is well recognized that the efficacy of antibiotics does not depend exclusively on their bacteriostatic or bactericidal effect on the bacteria, but also on reducing or inhibiting their virulence. In some clinical situations where the released toxins cause

severe progression of the disease, antimicrobials which inhibit toxin production are the preferred treatments (Micek *et al.*, 2005). In addition, since the possibility of subinhibitory concentrations of antibiotics in clinical situations is high, much work has been conducted to explore the effect of sub-MIC on bacterial virulence. Much work has been conducted and is ongoing in the study of the effect of subinhibitory concentrations of antibiotics on the virulence of different microorganisms (Dickgiesser and Wallach, 1987; Gemmell and Ford, 2002; Bernardo *et al.*, 2004; Adhikari and Novick, 2005; Spreer *et al.*, 2007; Yanagihara *et al.*, 2008). Most of the work has been done for microorganisms in the planktonic state (Sonstein and Burnham, 1993; Wu *et al.*, 2002; Dal Sasso *et al.*, 2003; Galice *et al.*, 2006; Koszczol *et al.*, 2006). However, very little work has been conducted to evaluate the effect of sub-MICs on the secreted virulence factors from biofilms (Masadeh, 2005).

Proteases are one of the types of virulence factors produced by most strains of *S. aureus*. They act on bacterial proteins, as part of the protein processing steps, or degrade them as a tactic by which the pathogenic bacteria change their status from invasion to colonization. In addition, some proteases act on host proteins to help the pathogenic bacteria in invading the body tissues. However, the production of excreted proteases from *S. aureus* NCTC 11962 was not detected in the eluate supernatants (whether treated or control) when tested by both azocasein assay and zymography under the prescribed experimental conditions used in this project (Chapter 5, section 5.5.1). However, this does not conclude the complete absence of proteases in this organism. Karlsson and Arvidson (2002) found that all the clinical strains of *S. aureus* that were screened for protease activity including those that were thought to be protease negative, contain the major genes that encode proteases. Therefore, the variation in protease secretion was attributed to the degree of protease gene expression, which could be regulated by several factors including

the presence of protease gene repressors. Thus, based on this explanation, it can be concluded that the presence of sub-MICs of antibiotics did not induce protease gene expression in the experiments conducted in this project.

The production of coagulase is used to classify staphylococci as either coagulase positive or coagulase negative. *S. aureus* is almost the only coagulase positive *Staphylococcus* spp. isolated from human specimens (Mack *et al.*, 2006). Coagulase is believed to be a virulence factor (Jonsson *et al.*, 1985; Sawai *et al.*, 1997). When *S. aureus* biofilms were exposed to sub-MICs of the antibiotics, the bacterial response was variable with regard to coagulase production according to the challenging antibiotic (Chapter 5, section 5.2.1). Roxithromycin produced a significant reduction in coagulase activity (Chapter 5, section 5.2.2). This effect is predicted based upon the mechanism of action of this macrolide. Roxithromycin is a general protein synthesis inhibitor. It acts by inhibiting ribosome assembly, early dissociation of tRNA and blocking the progression of the polypeptide chain. Therefore, it would be reasonable to suggest that sub-MIC roxithromycin was able to inhibit one or more of the steps involved in coagulase synthesis. On the other hand, based on the mode of action of ciprofloxacin and cefalexin, it is expected that they do not have a direct effect on coagulase synthesis, thus, its production was comparable to that of untreated biofilm.

Another important virulence determinant in microorganisms is the ability to adhere and form biofilms. In general, what determines the effect of exposing bacteria to sub-MIC of antibiotics, in terms of their ability to adhere and to form biofilms, is the bacterial type and strain and concentration of the antibiotic (Shibl, 1985; Fonseca *et al.*, 2004; Majtan *et al.*, 2007; Starner *et al.*, 2008). For example, sub-MICs of cefotaxime have been found to significantly induce biofilm formation and exopolysaccharide production in *Salmonella typhimurium*, while sub-MICs of ciprofloxacin and gentamicin

have reduced biofilm formation and exopolysaccharide production (Majtan *et al.*, 2007). In another study, sub-MICs of gentamicin have been found to have no effect on biofilm formation of *Haemophilus influenzae*, while sub-MIC azithromycin has significantly decreased biomass and maximal thickness of these biofilms (Starner *et al.*, 2008). Fonseca *et al.*, (2004), have shown that piperacillin / tazobactam significantly decrease the adhesion of all strains of *Pseudomonas aeruginosa* used in their study. The reason was linked to the effect of sub-MICs of the antibiotic on other virulence factors of the organism, such as altering cell surface hydrophobicity, inhibition of motility and possible effects on surface adhesins (Fonseca *et al.*, 2004). The adherence of *Fusobacterium nucleatum*, a colonizer in dental biofilm, has been found to be reduced when exposed to sub-MICs of penicillin, chlorhexidine, triclosan, and metronidazole. The reduction was attributed to surface adhesins, as the hydrophobicity of the microbe was not altered (Okamoto *et al.*, 2002).

The ability of *S. aureus* to form biofilms in polystyrene microtiter plate wells was tested in the presence of sub-MICs of the selected antibiotics and in controls (Chapter 5, section 5.3.1). The results exhibit that cefalexin, in the range of 2.81% to 45% MIC has a stimulatory or potentiating effect on bacterial adhesion and biofilm formation, while ciprofloxacin and roxithromycin do not exhibit such an effect (Chapter 5, section 5.3.3). Cefalexin is a cell wall synthesis inhibitor. Therefore, it can be assumed that its effect at sub-MIC could have affected the cell surface of the bacteria in favour of increased hydrophobicity, that has led to increased adherence. A similar increase in hydrophobicity was found in *Staphylococcus epidermidis* when studied in a continuous ambulatory peritoneal dialysis *in vitro* model (Hanlon *et al.*, 2004). The adherence of the organism to the surface of catheters and its colonization and formation of a biofilm has been linked to this increase in hydrophobicity (Hanlon *et al.*, 2004). The other explanation for the

stimulatory effect of cefalexin on biofilm formation is that, cefalexin could exhibit an indirect induction of biofilm formation through stimulation of α -toxin production. α -toxin has been found to be an essential compound for cellular interaction in the second phase of biofilm formation (Caiazza and O'Toole, 2003). β -lactams at subinhibitory concentrations have been found to induce α -toxin production (Gemmell, 1995; Ohlsen *et al.*, 1998; Stevens *et al.*, 2007). Therefore, cefalexin stimulatory effect on biofilm formation could be attributed to α -toxin induction. Biofilm enhancing effect of β -lactams in staphylococci have been shown in some studies (Dunne, 1990; Frank *et al.*, 2007) and their inhibitory effect on biofilm formation has been shown in other studies (Dunne, 1990; Rupp and Hamer, 1998). These variations could be ascribed to the type of β -lactam used, its concentration and the bacterial strain. On the other hand, protein synthesis inhibitors have variable effects on biofilm formation in staphylococci: some induce biofilm formation and others inhibit it (Rachid *et al.*, 2000; Frank *et al.*, 2007).

Biofilm production at different sub-MICs of roxithromycin was comparable to that of control (Chapter 5, section 5.3.3). This suggests that sub-MICs of roxithromycin were neither stimulatory (potentiating) nor inhibitory, except at 90% MIC, where no biofilm was formed. Since roxithromycin is a protein synthesis inhibitor, this could imply that none of the effector agents that are synthesized and involved in biofilm production were inhibited by roxithromycin in the range 0.35% to 45% MIC. Therefore, biofilm formation was not inhibited. At 90% MIC the inhibition of biofilm formation could be a result of inhibiting regulatory proteins required for biofilm formation. On the other hand, it can also be postulated that none of the repressors to biofilm formation were inhibited by roxithromycin (0.35% to 90% MIC), therefore, biofilm formation was not induced.

Sub-MIC ciprofloxacin did not affect biofilm formation in *S. aureus* (Chapter 5, section 5.3.3). Although this was true for other fluoroquinolones at sub-MICs when *S.*

epidermidis was exposed to them (Perez-Geraldo *et al.*, 1994; Rupp and Hamer, 1998), however, fluoroquinolones were found to have an inhibitory effect when *P. aeruginosa* was exposed to them (Yassien *et al.*, 1995). Similarly, sub-MICs of ciprofloxacin have resulted in a reduction in the number of bacteria attached to epithelial cells, where the reason was attributed to a reduction in the hydrophobicity of the bacteria (Wojnicz and Jankowski, 2007). These results indicate that the effect of sub-MICs of antibiotics on microorganisms is species and strain dependent.

7.4 Effect of sub-MIC of antibiotics on TSST-1 production and biological activity

As was discussed earlier, sub-MIC of antibiotics have variable effects on the production of bacterial virulence factors. For TSST-1, one of the aims of this thesis was to study the effect of 1/16 MIC of selected antibiotics on the production and biological activity of TSST-1 released from biofilms. In the literature, after identifying the biological activity of TSST-1 in the early stages after its discovery (Calvano *et al.*, 1984; Micusan *et al.*, 1986; Uchiyama *et al.*, 1987; Parsonnet and Gillis, 1988) TSST-1 biological activity studies have been continued in order to study the mechanism of host response under different variables (See *et al.*, 1992; Akatsuka *et al.*, 1994; Davison *et al.*, 2000; Brandt *et al.*, 2002), to identify TSST-1 structural domains or regions role and activity (Murray *et al.*, 1996; Wahlsten and Ramakrishnan, 1998) and to conduct structure-function analysis mainly by studying TSST-1 mutants (Blanco *et al.*, 1990; Bonventre *et al.*, 1993; Stiles *et al.*, 1995). However, no previous or current studies have been conducted to observe the effect of sub-MICs of antibiotics on the biological activity of TSST-1 released from biofilms of *S. aureus* when exposed to these antibiotics.

TSST-1 was extracted and purified from eluate supernatants of biofilms that were exposed to 1/16 MIC of antibiotics and control (Chapter 6, section 6.2). The proliferative activity of TSST-1 was evaluated on mouse splenocytes (Chapter 6, section 6.3). Ten-fold serial dilutions were prepared from each purified toxin and the proliferative activities were compared for a concentration range from 0.1 to 1000ng mL⁻¹. It was found that TSST-1 is active in this range, but with an optimal activity at 100ng mL⁻¹ (Chapter 6, Figure 6.7). The presence of optimal activity for mitogens was also noticed when phytohaemagglutinin-P (PHA-P) was tested (Chapter 6, Figure 6.6), where 10µg mL⁻¹ was the optimum concentration above which a reduction in the response was obtained. In another study, concanavalin A produced similar effects when applied to splenocytes (Lyu and Park, 2006). Similar findings have been obtained by Gampfer *et al.*, (2002). In their study, the optimal proliferative response of human peripheral mononuclear cells to TSST-1 in the range of 1 to 500ng mL⁻¹ in one experiment and of 0.01 to 1000 ng mL⁻¹ in another experiment was at 100ng mL⁻¹. After that a reduction in response was observed. As shown in Figure 6.7 and Table 6.1, at each concentration level, there is no significant difference in the response of splenocytes to TSST-1 purified from control or from eluate supernatants exposed to treatments, except the one at 1000ng mL⁻¹. This indicates that exposing *S. aureus* biofilms to 1/16 MIC of cefalexin or ciprofloxacin has no effect on the proliferative activity of the toxin.

On the other hand, the proliferative activity of ten fold serial dilutions of crude eluate supernatants of the control and treated ones, resulted in a similar pattern of responses, where the response was dose dependent and has an optimum after which a reduction was observed (Chapter 6, Figure 6.8). In addition, an interesting response was observed when the proliferative activity of TSST-1 purified from eluate supernatants was compared with the proliferative activity of crude eluate supernatants exposed to sub-MIC

antibiotics at equivalent TSST-1 concentrations (Figures 6.7 and 6.8). Although it would be reasonable to accept that toxin purification and concentration steps might have some negative effect in terms of proliferative activity. However, the remarkable difference in activity (Figures 6.7 and 6.8) in terms of OD % from PHA-P, could be attributed to a mitogenic agent present in the crude eluate supernatant, but of course, not in the purified TSST-1.

S. aureus NCTC 11962 produces enterotoxin A. This toxin is known to have superantigenic activity (Lee and Bohach, 2004). It acts in a similar way to TSST-1 by binding to the T-cell receptor. While TSST-1 stimulates V β 2 and V β 5.1, enterotoxin A stimulates a family of V β chains known as (V β 5.2/3) (Saloga *et al.*, 1996; Davison *et al.*, 2000). Enterotoxin A has a molecular weight of 27.5kDa and has been reported to have multiple isoelectric points (8.1, 7.68, 7.26, 6.64; Schantz *et al.*, 1972). The two major forms obtained by Reynolds *et al.* (1988), were pI 8.1 and 7.08. By referring back to eluate supernatant 2D-gels (Chapter 4; Figures: 4.14, 4.15, 4.16, 4.17; Tables: 4.13, 4.14, 4.15, 4.16) a spot appearing at M_r 26.5kDa and pI 8.27 (spot 6) is most probably enterotoxin A. Therefore, the presence of another mitogenic agent in crude eluate supernatants may explain the higher response of splenocytes to crude eluates than to equivalent concentrations of purified TSST-1. However, due to time considerations, a decision was taken not to further investigate this toxin during this project.

Generally, it is recognized that protein synthesis inhibitors inhibit the production of TSST-1 and other exoproteins (Schlievert and Kelly, 1984; Dickgiesser and Wallach, 1987; Herbert *et al.*, 2001). However, roxithromycin at 1/16 MIC has no effect on the production of TSST-1 from *S. aureus* biofilm exposed to it (Chapter 5, section 5.4). One of the reasons that could explain the discrepancy in results could be attributed to the mode of action of this macrolide on TSST-1, as it is known that there are several mechanisms of

action of protein synthesis inhibitors. The other reason could be the concentration of antibiotic used in this thesis. For example, tetracycline at 1/4 MIC reduced the synthesis of TSST-1, while 1/16 MIC has no effect (Dickgiesser and Wallach, 1987). Therefore, the inhibitory effect of roxithromycin on TSST-1 production can not be ruled out at higher concentrations.

1/16 MIC of Ciprofloxacin resulted in reduction to TSST-1 production from biofilms exposed to it. This effect was also demonstrated by Parsonnet *et al.*, (1994) where they showed that fluoroquinolones, clindamycin and rifampin suppress TSST-1 production up to 90%.

Cefalexin was found to reduce the production of TSST-1 from *S. aureus* biofilms when exposed to 1/16 MIC of cefalexin (Chapter 5, Table 5.7). However, in the literature the effect of β -lactams on TSST-1 production is variable. For example ampicillin, even at relatively high concentrations has no effect on TSST-1 production (Schlievert and Kelly, 1984), while sub-MIC nafcillin increased toxin production (Parsonnet *et al.*, 1994; Stevens *et al.*, 2007). This effect was suggested to be related to the action of nafcillin on the cell wall resulting in increased cell wall permeability and rupture releasing the toxin outside the cell (Parsonnet *et al.*, 1994).

It is clear from studying the biological activity of purified TSST-1 (Chapter 6) that cefalexin at 1/16 MIC does not cause any alteration in the synthesis of the toxin that would lead to biological malfunctioning. This was found in Chapter 6 where the biological activity of purified toxins from biofilms exposed to sub-MIC cefalexin was not altered. At the same time, the amount of TSST-1 produced was reduced (Chapter 5, Table 5.7), which means that cefalexin has either caused a blockage in the TSST-1 synthesis pathway or an inhibition to its maturation and release from the cell. β -lactams are small molecules that can pass through the cell wall. Therefore, they have access to the inside of

the cell where the premature toxin is present. It is well known that β -lactams are analogues of the D-alanyl-D-alanine residue of the peptidoglycan of the cell wall, thus inhibiting the action of transpeptidases. In *S. aureus*, the signal peptidase enzyme acts on TSST-1 precursor to cleave the 40 amino-acid signal peptide, a step that occurs during or immediately after translocation across the cytoplasmic membrane (Sibbald *et al.*, 2006; Figure 7.1).

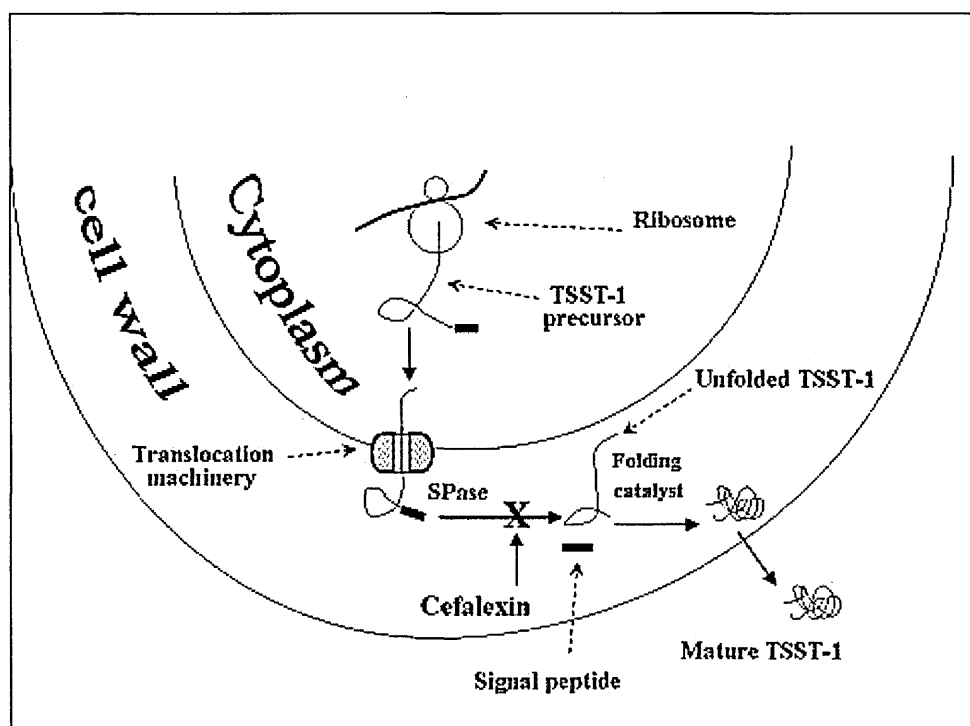
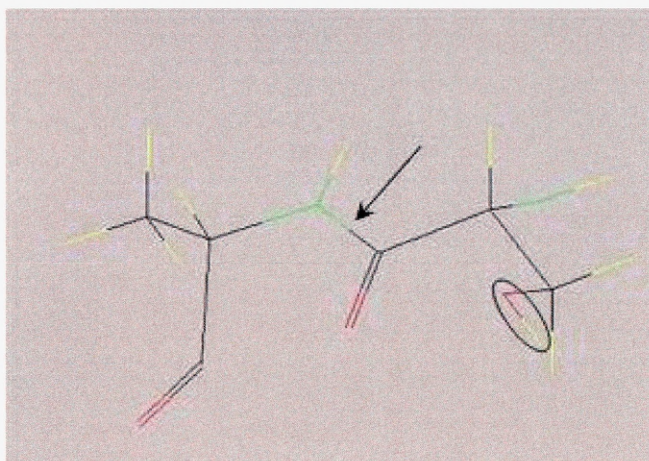


Figure 7.1: Synthesis and secretion of TSST-1 and the postulated inhibitory effect of cefalexin. When the nascent protein (TSST-1 precursor) is produced, it is accompanied by chaperones in the cytoplasm to the translocation machinery present on the cytoplasmic membrane. During or after translocation, signal peptidase (SPase) acts at the cleavage point of the precursor (ala-ser, amino-acids 40 & 41) thus, cleaving the signal peptide. The TSST-1, is now present in the periplasmic space in an unfolded state. Immediately the folding catalyst acts on the toxin and the mature toxin is released outside the cell since it has no retention signal (Sibbald *et al.*, 2006). It is postulated that cefalexin, at 1/16 MIC, binds to the active site of the signal peptidase as it is an analogue of the ala-ser residue in much the same way it is also an analogue of the ala-ala residue of peptidoglycans. Thus, inhibiting the activity of signal peptidase. Therefore, the process of releasing the toxin is inhibited.

The cleavage point of the signal peptide from the toxin is an alanine-serine residue (Blomster-Hautamaa *et al.*, 1986). Structurally, serine ($\text{HOCH}_2\text{CHNH}_2\text{COOH}$) is very

similar to alanine ($\text{CH}_3\text{CHNH}_2\text{COOH}$), it contains an additional hydroxyl group replacing one of the hydrogen atoms (Figure 7.2). This small molecular weight group (OH) is not expected to impose any extra steric hindrance to the alanine-serine residue when compared to an alanine-alanine residue. Therefore, depending on this structural similarity between alanine-alanine and alanine-serine (Figure 7.2), and based on the mode of action of β -lactams, it can be hypothesized that cefalexin is also an analogue of alanine-serine (amino-acids 40 and 41 of the TSST-1 precursor) and inhibits the signal peptidase by binding to it (Figure 7.1). According to this hypothesis, smaller amounts of TSST-1 will be cleaved from the precursor toxin in the presence of 1/16 MIC cefalexin. However, the increase in production of TSST-1 when *S. aureus* has been exposed to certain β -lactams (Parsonnet *et al.*, 1994; Stevens *et al.*, 2007) could be attributed to the types of β -lactam used, their concentrations, bacterial strain and the model of study used.

A)



B)

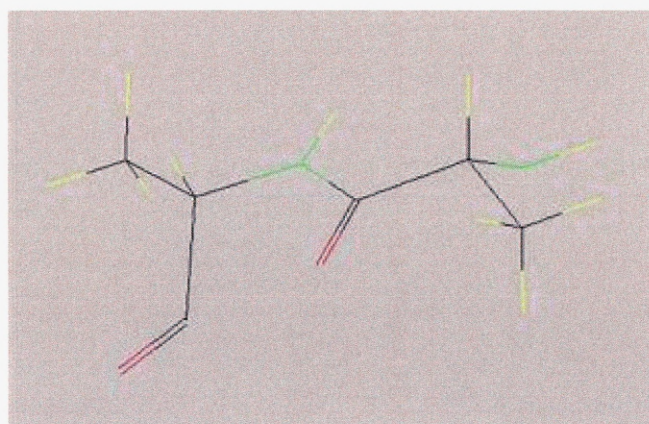


Figure 7.2: Similarity in structures between alanine-serine residue (A), that is the cleavage point in TSST-1 precursor (amino-acids 40 and 41) and alanine-alanine (B). The arrow indicates the cleavage point between the alanine (left) and the serine residue (right). Structurally, serine is an alanine with a hydroxyl (OH) group (circled) replacing one of the Hydrogen (H) atoms of the methyl (-CH₃) moiety. Key for the colours: yellow: **Hydrogen, green: **Nitrogen**, black: Carbon, red: **Oxygen**. Single lines represent single bonds between atoms and double lines represent double bonds.**

7.5 Conclusion

- In order to support the growth of staphylococci, in addition to the basic nutrients (organic carbon, nitrogen, iron, magnesium and sources of ions) several amino acids are required. Whereas complex media such as brain heart infusion is needed to support the growth of staphylococci in Sorbarod model biofilms.
- Exposing *Staphylococcus aureus* biofilms to sub-MICs as low as 1/16 MIC of cefalexin, ciprofloxacin and roxithromycin resulted in marked changes in protein profiles of cellular proteins and secreted ones.
- Secreted proteases were not detected in the eluates of *S. aureus* NCTC 11962 biofilms or those challenged with sub-MIC of selected antibiotics. However, the activity of soluble coagulase was reduced in the eluates of biofilms exposed to the protein synthesis inhibitor antibiotic, roxithromycin. Although it was not significantly affected by the other antibiotics.
- At a certain range of sub-MICs, cefalexin induced biofilm formation of *S. aureus* NCTC 11962, while the effect of sub-MICs of ciprofloxacin and roxithromycin treated cells was comparable to the untreated, except at 90% MIC of roxithromycin, where no biofilm was detected.
- TSST-1 production was significantly lower in eluates challenged with cefalexin than in control and other treatments, an effect that is hypothesized to be related to the inhibitory effect of cefalexin on signal peptidase. Thus, inhibiting the cleavage of TSST-1 precursor to produce TSST-1. On the other hand, exposing *S. aureus* biofilms to 1/16 MIC of the selected antibiotics did not affect the proliferative activity of the purified TSST-1

7.6 Suggestions for Future work

- SDS-PAGE and 2D-PAGE analyses, showed the appearance of novel proteins and disappearance of others when the *S. aureus* biofilm was exposed to sub-MIC of the selected antibiotics. Identifying these proteins using Mass spectrometry (MALDI-TOF), would provide more information about the nature and potential role of the proteins and their potential effect on bacterial virulence.
- It has been shown that cefalexin-induced biofilm formation at a certain range of sub-MICs, has no effect on biofilm formation at other sub-MICs. The reason was attributed to either changes in cell surface hydrophobicity or to stimulatory effect to α -toxin production, which is known to play a role in biofilm formation. These two suggestions could be tested at different sub-MICs of cefalexin using the same bacterial strain. Cell surface hydrophobicity can be determined for bacterial cells exposed to sub-MICs and control by either n-Hexadecane assay or by salt aggregation test of ammonium sulphate. α -toxin production can be assessed and compared between eluate supernatants challenged with antibiotics and control by testing the haemolysis of rabbit erythrocytes.
- The proliferative activity of crude eluate supernatants exposed to sub-MIC of antibiotics was more than that of purified TSST-1 when equivalent concentrations were tested. The reason was linked to the effect of enterotoxin A. Therefore, the effect of 1/16 MIC of cefalexin, ciprofloxacin and roxithromycin on the production and perhaps the activity of enterotoxin A should be elucidated. Enterotoxin A production can be semi-quantified by a method based on antigen-antibody reaction such as the one used in this work to quantify TSST-1 or ELISA method. Its activity can be determined by purifying the toxin and testing its proliferative activity.

- It was hypothesized that cefalexin at 1/16 MIC inhibits the cleavage of TSST-1 precursor through inhibiting signal peptidase. Therefore, TSST-1 production is reduced. This will necessarily result in accumulation of TSST-1 precursor in the cell. In order to test this hypothesis, the abundance of TSST-1 precursor relative to TSST-1 could be determined in challenged and control cells. To do so, protein spots of TSST-1 precursor and TSST-1 in 2D-gels loaded with equal amounts of protein can be measured by densitometer or can be excised, treated and quantified by mass spectroscopy.

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